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Günter Oberdörster, Eva Oberdörster, Jan Oberdörster

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An Emerging Discipline Evolving from Studies of Ultrafine Particles

Günter Oberdörster[1], Eva Oberdörster[2], Jan Oberdörster[3]

[1]University of Rochester

Department of Environmental Medicine

Rochester, NY

[2]Southern Methodist University

Department of Biology

Dallas, TX

[3]Bayer CropScience

Toxicology Department

Research Triangle Park, NC

Corresponding Author:

Dr. Günter Oberdörster

University of Rochester

Department of Environmental Medicine

575 Elmwood Avenue, MRBx Bldg., Box 850

Rochester, NY 14642 USA

e-mail: Gunter_Oberdorster@urmc.rochester.edu

fax: 585-256-2631; tele: 585-275-3804

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*(*The views expressed by the authors are their own and do not necessarily reflect those of the U.S. EPA)*

List of abbreviations

^{13}C	=	radiolabled Carbon
^{192}Ir	=	radiolabled Iridium
Al_2O_3	=	Aluminum oxide
ANSI	=	American Standards Institute
C5a	=	Complement protein 5a
C_{60}	=	fullerene (60)
Ca++	=	calcium

cm	=	centimeter
CMD	=	Count Median Diameter
CNS	=	Central Nervous System
CYP2K1	=	cytochrome P450 2K1
EPA	=	Environmental Protection Agency
EU	=	European Union
GSH	=	glutathione
hr	=	hour
IARC	=	International Agency for Research on Cancer
ICON	=	International Council on Nanotechnology
ICRP	=	International Commission on Radiological Protection
ILSI	=	International Life Sciences Institute
ISC	=	Inter System Crossing
kg	=	kilogram
LPO	=	lipid peroxidation
m	=	meter
□g	=	microgram
mg	=	milligram
□m	=	micrometers
mm	=	millimeter
MMAD	=	Mass Median Aerodynamic Diameter
Mn	=	Manganese
MPPD	=	Multiple Path Particle Deposition
MSDS	=	Material Safety Data Sheet
MWNT	=	multi-walled Carbon Nanotubes
ng	=	nanogram
nm	=	nanometer
NNI	=	National Nanotechnology Initiative
NP	=	engineered nanoparticles

NRC	=	National Research Council
NSP	=	nano-sized particles
P450s	=	cytochrome P450s
PNS	=	Peripheral Nervous System
ppb	=	parts per billion
PTFE	=	polytetrafluoroethylene
ROS	=	Reactive Oxygen Species
ssDNA	=	single-stranded DNA
SWNT	=	Single-walled Carbon Nanotube
TiO_2	=	titanium dioxide
UFP	=	ultrafine particle
UV	=	ultraviolet radiation
WHO	=	World Health Organization

Outline of Nanotoxicology manuscript

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ABSTRACT

Although humans have been exposed to airborne nano-sized particles (NSP; <100 nm) throughout their evolutionary stages, such exposure has increased dramatically over the last century due to anthropogenic sources. The rapidly developing field of nanotechnology is likely to become yet another source through inhalation, ingestion, skin uptake, and injection of engineered nanomaterials. Information about safety and potential hazards is urgently needed. Results of older biokinetic studies with NSP and newer epidemiologic and toxicologic studies with airborne ultrafine particles can be viewed as the basis for the expanding field of nanotoxicology, which can be defined as safety evaluation of engineered nanostructures and nanodevices. Collectively, some emerging concepts of nanotoxicology can be identified from the results of these studies: When inhaled, specific sizes of NSP are efficiently deposited by diffusional mechanisms in all regions of the respiratory tract. The small size facilitates uptake into cells, transcytosis across epithelial and endothelial cells into the blood and lymph circulation to reach potentially sensitive target sites such as bone marrow, lymph nodes, spleen, heart. Access to CNS and ganglia *via* translocation along axons and dendrites of neurons has also been observed. NSP penetrating the skin distribute *via* uptake into lymphatic channels. Endocytosis, and biokinetics are largely dependent on NSP surface chemistry (coating) and *in vivo* surface modifications. The greater surface area per mass compared to larger-sized particles of the same chemistry renders NSP more active biologically. This activity includes a potential for inflammatory and pro-oxidant, but also anti-oxidant, activity, which can explain early findings showing mixed results in terms of toxicity of NSP to environmentally-relevant species. Evidence of mitochondrial distribution and oxidative stress response following NSP endocytosis points to a need for basic research about their interactions with subcellular structures. Additional considerations for assessing safety of engineered NSP include careful selections of appropriate and relevant doses/concentrations, the likelihood of increased effects in a compromised organism, but also the benefits of possible desirable effects. An interdisciplinary team approach (*e.g.*, toxicology, materials science, medicine, molecular biology, and bioinformatics, to name a few) is mandatory for nanotoxicology research to arrive at an appropriate risk assessment.

1. *Introduction*

1.1 Naturally-Occurring and Anthropogenic Nano-Sized Particles

Exposures to airborne nano-sized particles (NSP, <100 nm) have been experienced by humans throughout their evolutionary stages, but it is only with the advent of the industrial revolution that such exposures have increased dramatically due to anthropogenic sources such as internal combustion engines, power plants, and many other sources of thermodegradation. And, most recently, the rapidly developing field of nanotechnology is likely to become yet another source for human exposures to NSP – engineered nanoparticles (NP) – by different routes, *i.e.*, inhalation, ingestion, dermal or even injection. Table 1 summarizes some of the natural and anthropogenic sources of NSP, the latter divided into unintentional and intentional sources.

Biologically-based or naturally-occurring molecules that are found inside organisms since the beginning of life can serve as model nano-sized materials. For example, biogenic magnetite is a naturally occurring nano-sized particle that occurs in many species ranging from Bacteria to Protozoa and to Animals (Blakemore 1975; Kirschvink et al. 2001). Biogenic magnetite has even been found in brains of humans (Dunn et al. 1995; Kirschvink et al. 1992; Schultheiss-Grassi et al. 1999), and has been associated with neurodegenerative diseases (Dobson 2001; Hautot et al. 2003). A biological-model of coated nanomaterials can be found in ferritin, which is an ~12 nm large iron storage protein that contains 5-7 nm sized hydrous ferric oxide-phosphate inside a protective protein shell (Donlin et al. 1998). Nano-sized materials, including fullerenes, occur naturally from combustion processes such as forest fires and volcanoes.

Obvious differences between unintentional and intentional anthropogenic NSP are the polydispersed and chemically complex nature (elemental, soluble, and volatile carbon compounds; soluble and poorly soluble inorganics (Cyrys et al. 2003; Hughes et al. 1998)) of the former, in contrast to the monodisperse and precise chemically engineered characteristics and solid form of the latter, generated in gas or liquid phase (NNI, 2004). However, despite these differences the same toxicological principles are likely to apply for NP, since not only size but a number of other particle parameters determine their biological activity. The multitude of interactions of these factors has yet to be assessed, and this article is an attempt to summarize our present knowledge.

NSP are variably called ultrafine particles (UFPs) by toxicologists (EPA 2004), Aitken mode and nucleation mode particles by atmospheric scientists (Kulmala 2004; NRC 1983), and engineered nanostructured materials by materials scientists (NNI 2004). Figure 1 depicts the range of sizes of airborne ambient particulate matter, including the nucleation mode, Aitken mode, accumulation mode and coarse mode particles. Ambient particles below 0.1 μm, defined as ultrafine particles in the toxicological literature, consist of transient nuclei or Aitken nuclei (NRC 1983). More recently, even smaller particles in the nucleation mode with peak diameters around 4 nm have been observed (McMurry and Woo 2002). The distinction between NSP generated by internal combustion engines and engineered nanoparticles becomes further clouded by the finding of nanotubes in diesel exhaust (Evelyn et al. 2003). The use of the term "nano" in this review only reflects on particle size and not on chemical composition. For the purposes of this review, we will use the following terms: "nano-sized particle" includes all engineered and ambient nano-sized spherical particles below 100 nm. "Engineered nanoparticles" (NP) includes only spherical NSP specifically engineered in the laboratory; other engineered nano-sized structures will be labeled according to their shape, *e.g.*, nanotubes, -fibers, -wires, -rings, *etc.* "Ultrafine Particles (UFP)" includes ambient and laboratory-generated NSP that are not produced in a controlled, engineered way.

Table 2 shows the tremendous differences in particle number concentrations and particle surface areas for particles of the four ambient modes, assuming an airborne concentration of 10 $\mu g/m^3$ of unit density particles of each size. The extraordinarily high number concentrations of NSP per given mass will likely be of toxicological significance when these particles interact with cells and subcellular components. Likewise, their increased surface area per unit mass can be toxicologically important if other characteristics like surface chemistry and bulk chemistry, are the same. Although the mass of UFP in ambient air is very low, approaching only 0.5 - 2 $\mu g/m^3$ at background levels (Hughes et al. 1998), it can increase several-fold during high pollution episodes or on highways (Brand et al. 1991; Shi et al. 2001; Zhu et al. 2002).

1.2 Physico-Chemical Characteristics as Determinants of Biological Activity

The small size and corresponding large specific surface area of solid NSP (Table 2) confers specific properties to them, for example, making them desirable as catalysts for chemical reactions. The importance of surface area becomes evident when considering that surface atoms

or molecules play a dominant role in determining bulk properties (Amato 1989); the ratio of surface to total atoms or molecules increases exponentially with decreasing particle size (Figure 2). Increased surface reactivity predicts that NSP exhibit greater biological activity per given mass compared to larger particles, should they be taken up into living organisms and provided they are solid rather than solute particles. This increased biological activity can either be positive and desirable (*e.g.*, antioxidant activity, carrier of capacity for therapeutics, penetration of cellular barriers for drug delivery), or negative and undesirable (*e.g.*, toxicity, induction of oxidative stress or of cellular dysfunction) or a mix of both. Not only may adverse effects be induced, but interactions of NSP with cells and subcellular structures and their biokinetics are likely to be very different from those of larger-sized particles. For example, virologists have described more than 60 years ago the translocation of 30-50 nm sized virus particles along axons and dendrites of neurons and across epithelia (Bodian and Howe 1941), whereas first reports about increased inflammatory activity and epithelial translocation of man-made 20 and 30 nm solid particles appeared only more recently (Ferin et al. 1990; Oberdörster et al. 1990).

The characteristic biokinetic behaviors of NP are attractive qualities for promising applications in medicine as diagnostic and therapeutic devices, and as tools to investigate and understand molecular processes and structures in living cells (Akerman et al. 2002; deLorenzo 1970; Foley et al. 2002; Kreuter 2001; Li et al. 2003). For example, targeted drug delivery to tissues which are difficult to reach (*e.g.*, CNS), NP for the fight against cancer, intravascular nanosensor and nanorobotic devices, diagnostic and imaging procedures are presently under development. The discipline of nanomedicine –defined as medical application of nanotechnology and related research – has arisen to design, test, and optimize these applications so that they can eventually be used routinely by physicians (Freitas 1999).

However, in apparent stark contrast to the many efforts aimed at exploiting desirable properties of NP for improving human health are the limited attempts to evaluate potential undesirable effects of NP when administered intentionally for medicinal purposes, or following unintentional exposure during manufacture or processing for industrial applications: The same properties that makes NP so attractive for development in nanomedicine and for specific industrial processes could also prove deleterious when NP interact with cells. Thus, evaluating the safety of NP should be of highest priority given their expected world-wide distribution for industrial applications and the likelihood of human exposure, directly or through release into the

environment (air, water, soil). An emerging discipline – nanotoxicology, which can be defined as "Science of engineered nanodevices and nanostructures that deals with their effects and problems involved" — is gaining increased attention. Nanotoxicology research will not only provide data for safety evaluation of engineered nanostructures and devices but will also help to advance further the field of nanomedicine by providing information about their undesirable properties and means to avoid them.

1.3 Human Exposure to Nano-Sized Materials

In addition to natural and anthropogenic sources of UFP in the ambient air, certain workplace conditions also generate NSP which can reach much higher exposure concentrations, up to several hundred $\mu g/m^3$, than ambient levels. In contrast to airborne UFP exposures occurring via inhalation at the workplace and from ambient air, not much is known about levels of exposure *via* different routes for NP, whether it is by direct human exposure or indirectly through contamination of the environment. For example, are there or will there be significant exposures to airborne singlet engineered carbon nanotubes or C_{60} fullerenes? First measurements at a model workplace gave only very low concentrations, less than 50 $\mu g/m^3$, and these were most likely in the form of aggregates (Maynard et al. 2004). However, even very low concentrations of nano-sized materials in the air represent very high particle number concentrations, as is well known from measurements of ambient ultrafine particles (Hughes et al. 1998), For example, a low concentration of 10 $\mu g/m^3$ of unit density 20 nm particles translates into more than $1x10^6$ particles/cm^3 (Table 2). Inhalation may be the major route of exposure for NP, yet ingestion and dermal exposures also need to be considered during manufacture, use, and disposal of engineered nanomaterials, and specific biomedical applications for diagnostic and therapeutic purposes will require i.v., s.c. or i.m. administration (Table l). It can be assumed, though, that the toxicology of NP can build on the experience and data already present in the toxicology literature of ambient UFP.

(See web-materials for additional details.)

1.4 Manufactured Nanomaterials in the Environment

Manufactured nanomaterials are likely to enter the environment for several reasons: i) some are and others will be produced by the ton, and some of any material produced in such mass

quantities is likely to reach the environment from manufacturing effluent or from spillage during shipping and handling; ii) they are being used in personal-care products such as cosmetics and sunscreens and can, therefore, enter the environment on a continual basis from washing off of consumer products (Daughton and Ternes 1999); iii) they are being used in electronics, tires, fuel cells, and many other products and it is unknown whether some of these materials may leak out or be worn off over the period of use; iv) they are being used in disposable materials such as filters and electronics and may therefore reach the environment through landfills and other methods of disposal.

Scientists have also found ways of using nanomaterials in remediation. Although many of these are still in testing stages (Chitose et al. 2003; Jaques and Kim 2000; Joo et al. 2004; Nagaveni et al. 2004; Nghiem et al. 2004; Tungittiplakorn et al. 2004), dozens of sites have already been injected with various nanomaterials, including nano-iron (Mach 2004). Testing to determine the safety of these NP used for remediation to environmentally-relevant species has not yet been done. Although most people are concerned with effects on large wildlife, the basis of many food chains depends on the benthic and soil flora and fauna, which could be dramatically impacted by such NP injections. In addition, as noted by Lecoanet and Wiesner (2004a), nano-sized materials may not migrate through soils at rapid enough rates to be valuable in remediation. Future laboratory and field trials will help clear up the line between remediation and contamination.
(See web-materials for additional details.)

2. Toxicology of Airborne Ultrafine Particles, Overview

In recent years, interest in potential effects of exposure to airborne UFP has increased considerably, and studies have shown that they can contribute to adverse health effects in the respiratory tract as well as in extrapulmonary organs. Results on direct effects of ambient and model UFP have been reported from epidemiological studies and controlled clinical studies in humans, inhalation/instillation studies in rodents, or *in vitro* cell culture systems. For example, several epidemiological studies have found associations of ambient UFP with adverse respiratory and cardiovascular effects resulting in morbidity and mortality in susceptible parts of the population (Pekkanen et al. 1997; Penttinen et al. 2001; Peters et al. 1997a, b; von Klot et al. 2002; Wichmann et al. 2002), whereas other epidemiological studies have not seen such

associations (Pekkanen et al. 1997; Tiittanen et al. 1999). Controlled clinical studies evaluated deposition and effects of laboratory-generated UFP. High deposition efficiencies in the total respiratory tract of healthy subjects were found, and deposition was even greater in asthmatic and COPD subjects. In addition, effects on the cardiovascular system including blood markers of coagulation and systemic inflammation and pulmonary diffusion capacity were observed following controlled exposures to ultrafine carbonaceous particles (Anderson et al. 1990; Wichmann et al., 2000; Brown et al. 2002; Chalupa et al. 2004; Jaques and Kim 2000; Pietropaoli et al. 2004; Pekkanen et al., 2002; Henneberger et al., 2005).

Studies in animals using laboratory-generated model UFP or ambient UFP showed that UFP consistently induced mild yet significant pulmonary inflammatory responses as well as effects in extrapulmonary organs. Animal inhalation studies included the use of different susceptibility models in rodents, with analysis of lung lavage parameters and lung histopathology, effects on the blood coagulation cascade and translocation studies to extrapulmonary tissues (Elder et al. 2000; Elder et al. 2002, 2004; Ferin et al. 1991; Ferin and Oberdörster 1992; Kreyling et al. 2002; Li et al. 1999; Nemmar et al. 1999; Nemmar et al. 2002a; Nemmar et al. 2002b; Nemmar et al. 2003; Oberdörster et al. 1992a; Oberdörster et al. 1995; Oberdörster et al. 2000; Oberdörster et al. 2002; Oberdörster et al. 2004; Semmler et al. 2004; Zhou et al. 2003).

In vitro studies using different cell systems showed to varying degrees pro-inflammatory and oxidative stress–related cellular responses after dosing with laboratory-generated or filter collected ambient UFP (Brown et al. 2000; Brown et al. 2001; Li et al. 2003). Collectively, the *in vitro* results have identified oxidative stress related changes of gene expression and cell signaling pathways as underlying mechanisms of UFP effects, as well as a role of transition metals and certain organic compounds on combustion generated UFP (Figure 3). These can alter cell signaling pathways, including Ca++ signaling and cytokine signaling (*e.g.*, IL-8) (Donaldson et al., 2002; Donaldson and Stone, 2003). Effects were on a mass basis greater for ultrafine model particles than for those of fine particles, whereas for ambient UFP cellular responses sometimes were greater and sometimes less than those of fine and coarse particles. The interpretation of the *in vitro* studies is oftentimes difficult because particles of different chemical compositions were used, target cells were different, duration, endpoints, and generally high dose levels also differed. Results from high doses in particular should be viewed with caution if they are orders of magnitude higher than predicted from relevant ambient exposures (see section 3.4).

(See web-materials for additional details.)

3. Concepts of Nanotoxicology

3.1 Laboratory Rodent Studies

With respect to potential health effects of NSP, two examples should serve to illustrate *i*) that these particles have a higher inflammatory potential per given mass than larger particles, provided they are chemically the same, and *ii*) that UFP generated under certain occupational conditions can elicit severe acute lung injury.

The first example involves studies with ultrafine and fine TiO_2 particles which showed that ultrafine anatase TiO_2 (20 nm), when instilled intratracheally into rats and mice, induced a much greater pulmonary-inflammatory neutrophil response (determined in the lung lavage 24 hrs. after dosing) than fine anatase TiO_2 (250 nm) at the same instilled mass dose of both types of particles (Figure 4a). However, when the instilled dose was expressed as particle surface area it became obvious that the neutrophil response in the lung for both ultrafine and fine TiO_2 fitted the same dose-response curve (Figure 4b), suggesting that particle surface area for particles of different sizes but of the same chemistry, such as TiO_2, is a better dosemetric than particle mass or particle number (Oberdörster 2000). Moreover, normalizing the particle surface dose to lung weight shows excellent agreement of the inflammatory response in both species (Figure S-2 on web). The better fit of dose-response relationships by expressing the dose as surface area rather than mass when describing toxicological effects of inhaled solid particles of different sizes has been pointed out repeatedly, especially when particles of different size ranges – nano to fine – were studied (Brown et al. 2001; Donaldson et al. 1998; Donaldson et al. 2002; Oberdörster and Yu 1990; Oberdörster et al., 1992; Driscoll, 1996; Tran et al. 1998; Tran et al. 2000) (see web for additional material).

Particle chemistry, and specifically surface chemistry, play a decisive role in addition to particle size as is shown in the second example, *i.e.*, exposure of rats to polytetrafluoroethylene fume (PTFE). PTFE fume (generated by heating PTFE) has long been known to be of high acute toxicity to birds and mammals, including humans (Cavagna et al. 1961; Coleman et al. 1968; Griffith et al. 1973; Nuttall et al. 1964; Waritz and Kwon 1968). Analysis of these fumes revealed the nano-sized nature of the particles generated by heating PTFE to about 480°C, with a count median diameter (CMD) of 18 nm. They were highly toxic to rats, causing severe acute

lung injury with high mortality within 4 hours after a 15-min. inhalation exposure to 50 $\mu g/m^3$ (Oberdörster et al. 1995) This short exposure resulted in an estimated deposited dose in the alveolar regions of only ~60 ng. In humans, acute lung injury, known as polymer fume fever, can result from exposures to PTFE fumes (Auclair et al. 1983; Goldstein et al. 1987; Lee et al. 1997; Williams et al. 1974; Woo et al. 2001). Additional rat studies showed that the gas phase alone was not acutely toxic and that aging of the PTFE fume particles for 3 minutes increased their particle size to >100 nm due to accumulation which resulted in a loss of toxicity (Johnston et al. 2000). However, it is most likely that changes in particle surface chemistry during the aging period contributed to this loss of toxicity, and that this is not just an effect of the accumulated larger particle size. (Additional information on web).

These examples seem to represent the extremes of NSP in terms of pulmonary toxicity, one (TiO_2) being rather benign yet still inducing significantly greater inflammatory effects on a mass basis than fine particles of the same chemical make-up; the other (PTFE fumes) inducing very high acute toxicity, possibly related to reactive groups on the large surface per unit mass.

Engineered nanomaterials can have very different shapes, *e.g.*, spheres, fibers, tubes, rings, planes. Toxicological studies of spherical and fibrous particles have well established that natural (*e.g.*, asbestos) and man-made (*e.g.*, biopersistent vitreous) fibers are associated with increased risks of pulmonary fibrosis and cancer following prolonged exposures (Greim et al. 2001; IARC 2002). Critical parameters are the three D's: Dose, Dimension and Durability of the fibers. Fibers are defined as elongated structures with a diameter to length ratio (aspect ratio) of 1:3 or greater and with a length of >5 μm and diameter $\leq$ 3 μm (WHO 1985). Carbon nanotubes have aspect ratios of up to 100 and greater, and length can exceed 5 μm with diameters ranging from 0.7 to 1.5 nm for single-walled nanotubes, and 2 nm to 50 nm for multi-walled nanotubes. Results from three studies using intratracheal dosing of carbon nanotubes in rodents indicate significant acute inflammatory pulmonary effects which either subsided in rats (Warheit et al. 2004) or were more persistent in mice (Lam et al. 2004; Shvedova et al. 2004b). Administered doses were very high, ranging from 1 to 5 mg/kg in rats, and in mice from 3.3 to 16.6 mg/kg (Lam et al. 2004) or somewhat lower from 0.3 to 1.3 mg/kg (Shvedova et al. 2004b). Granuloma formation as a normal foreign body response of the lung to high doses of a persistent particulate material was a consistent finding in these studies. Metal impurities (e.g., Fe) from the nanotube generation process may also have contributed to the observed effects. Although these in vivo first

studies revealed high acute effects, including mortality, this was explained by the large doses of the instilled highly aggregated nanotubes which caused death by obstructing the airways and should not be considered a nanotube effect *per se* (Warheit et al. 2004). *In vitro* studies with carbon nanotubes also reported significant effects. Dosing keratinocytes and bronchial epithelial cells *in vitro* with single walled carbon nanotubes resulted in oxidative stress, as evidenced by the formation of free radicals, accumulation of peroxidative products, and depletion of cell antioxidants (Shvedova et al. 2004a). Multiwalled carbon nanotubes showed proinflammatory effects and were internalized in keratinocytes (Monteiro-Riviere et al. 2005). Again, relatively high doses applied in these studies need to be considered when discussing the relevancy of these findings for *in vivo* exposures. A most recent study in macrophages comparing single walled and multi-walled carbon nanotubes with C_{60} fullerenes found a cytotoxicity ranking on a mass basis in the order SWNT>MWNT>C_{60}. Profound cytotoxicity (mitochondrial function, cell morphology, phagocytic function) was seen for SWNT even at a low concentration of 0.38 $\mu g/cm^2$. The possible contribution of metal impurities of the nanotubes still needs to be assessed. Therefore, whether the generally recognized principles of fiber toxicology apply to these nanofiber structures needs still to be determined (Huczko et al. 2001).

Future studies should be designed to investigate both effects and also the fate of nanotubes following deposition in the respiratory tract, preferentially by inhalation using well dispersed (singlet) airborne nanotubes. In order to design the studies using appropriate dosing, it is necessary to assess the likelihood and degree of human exposure. It is of utmost importance to characterize human exposures in terms of the physico-chemical nature, the aggregation state and concentration (number; mass; surface area) of engineered nanomaterials and perform animal and in vitro studies accordingly. If using direct instillation into the lower respiratory tract a large range of doses, which include expected realistic exposures of appropriately prepared samples, needs to be considered.
(See web-materials for additional details.)

3.2 Ecotoxicological Studies:

Studies have been carried out to date only on a few species that have been accepted by regulatory agencies as models for defining ecotoxicological effects. Tests with un-coated, water soluble, colloidal fullerenes (nC_{60}) show that the 48 hour LC_{50} in *Daphnia magna* is 800 ppb

(Oberdörster E, 2004), using standard EPA protocols (EPA 1994). In largemouth bass (*Micropterus salmoides*), although no mortality was seen, lipid peroxidation in the brain and glutathione depletion in the gill was observed after exposure to 0.5 ppm nC_{60} for 48 hours (Oberdörster 2004). There are several hypotheses as to how lipid damage may have occurred in the brain, including direct redox activity by fullerenes reaching the brain *via* circulation or axonal translocation (see also section 4.1.2) and dissolving into the lipid-rich brain tissue; oxyradical production by microglia; or reactive fullerene metabolites may be produced by cytochrome P450 metabolism. Initial follow-up studies using suppressive subtractive hybridization of pooled control fish *vs.* pooled 0.5 ppm-exposed fish liver mRNA were also performed. Proteins related to immune responses and tissue repair were up-regulated, and several proteins related to homeostatic control and immune control were down-regulated. A cytochrome P450 (CYP2K1) involved in lipid metabolism was up-regulated. (See web-materials for additional details).

In addition to these biochemical and molecular-level changes in fish, bactericidal properties of fullerenes have also been reported, and are being explored as potential new anti-microbial agents (Yamakoshi et al. 2003). Engineered nanomaterials used as anti-microbials may shift microbial communities if they are released into the environment *via* effluents. As we know from anthropogenic Endocrine Disrupting Compounds, interference of signaling between nitrogen-fixing bacteria and their plant hosts could be extremely harmful both ecologically and economically in terms of crop production (Fox et al. 2001).

Aqueous fullerenes and coated SWCNT are stable in salt solutions (Cheng et al. 2004; Warheit et al. 2004), cell culture media (Lu et al. 2004; Sayes et al. 2004), Reconstituted Hard Water and MilliQ water (Dieckmann et al. 2003; Oberdörster 2004). NSP will tend to sorb onto sediment and soil particles and be immobilized due to their high surface area:mass ratio (Lecoanet and Wiesner 2004b). Biological transport would occur from ingested sediments, and one would expect movement of nanomaterials through the food chain (Figure 5).

To make engineered nanomaterials more biocompatible, both surface coatings and covalent surface modifications have been incorporated. Some studies have shown that both the surface coating and the covalent modifications can be weathered by either exposure to the oxygen in air or by UV irradiation for one to four hours (Derfus et al. 2004; Rancan et al. 2002). Therefore, although coatings and surface modifications may be critically important in drug-delivery devices, the likelihood of weathering under environmental conditions makes it important

to study toxicity under UV and air exposure conditions. Even coatings used in drug delivery of NP may not be biopersistent or could be metabolized to expose the core NP material. (See web-materials for additional details.)

3.3 ROS Mechanisms of Nano-sized Particle Toxicity

Both *in vivo* and *in vitro*, NSP of various chemistries have been shown to create reactive oxygen species (ROS). ROS production has been found in NP as diverse as C_{60} fullerenes, SWNT, quantum dots, and UFP, especially under concomitant exposure to light, UV or transition metals. (Brown et al. 2000; Brown et al. 2001; Derfus et al. 2004; Joo et al. 2004; Li et al. 2003; Nagaveni et al. 2004; Oberdörster 2004; Rancan et al. 2002; Sayes et al. 2004; Shvedova et al. 2004b; Wilson et al. 2002; Yamakoshi et al. 2003). It has been demonstrated that NSP of various sizes and various chemical compositions preferentially mobilize to mitochondria (DeLorenzo 1970; Gopinath et al. 1978; Foley et al. 2002; Li et al. 2003; Rodoslav et al. 2003). Since mitochondria are redox active organelles, there is a likelihood of altering ROS production and thereby over-loading or interfering with anti-oxidant defenses (Fig. 3).

Figure 6 diagrams some of the anti-oxidant defense systems that occur in animals, and possible areas where NSP may create oxyradicals. The C_{60} fullerene is shown as a model NP producing superoxide, as has been shown by Yamakoshi (2003) (Yamakoshi et al. 2003). The exact mechanism by which each of these diverse NP cause ROS is not yet fully understood, but suggested mechanisms include i) photo excitation of fullerenes and SWNT causing Inter-System Crossing (ISC) to create free electrons; ii) metabolism of NP to create redox active intermediates, especially if metabolism is *via* cytochrome P450s; iii) inflammation responses *in vivo* may cause oxyradical release by macrophages. Likely other mechanisms will emerge as studies on NP toxicity continue.

The small size and respective large specific surface area of NP, like those of ambient airborne UFP, gives them unique properties with respect to a potential to cause adverse effects. Certainly, as we learned from studies with UFP, chemical composition and other particle parameters are additional important effect modifiers. Results from these studies will, therefore serve as a basis for future studies in the field of nanotoxicology, for example, the propensity of NSP to translocate across cell layers and along neuronal pathways (see section 4.1.2).

3.4 Exposure Dose–Response Considerations:

A careful evaluation of exposure-dose-response relationships is critical to the toxicological assessment of NSP. This includes not only questions about the dosemetric – mass or number or surface of the particles as discussed before - but most importantly also the relevance of dose levels. For example, it is tempting, and continuously being done, to dose primary cells or cell-lines *in vitro* with very high doses without any consideration or discussion of realistic *in vivo* exposures; for instance, 100 μg NSP per ml of culture medium – labeled as a low dose – is extremely high and is unlikely to be encountered *in vivo*. Likewise, intratracheally instilling several hundred μg into a rat does not resemble a relevant *in vivo* inhalation exposure, both dose and dose rate cause high bolus dose artifacts. While such studies may be used in a first proof of principle approach, it is mandatory to follow up and validate results using orders of magnitude lower concentrations resembling realistic *in vivo* exposures, including worst case scenarios. The 500 year-old phrase "*the dose makes the poison*" can also be paraphrased as "*the dose makes the mechanism*": Mechanistic pathways operating at low realistic doses are likely to be different at very high doses when the cell's or organism's defenses are overwhelmed.

Therefore, in vivo and *in vitro* studies will only provide useful data on the toxicity and mode of action of NSP provided that justifiable concentration/doses are considered when designing such studies. This approach is particularly important for the proper identification of the dose-response curve. When data are generated only at high concentrations/doses, it is difficult to determine whether the dose response curve in question is best described by a linear (no threshold), supralinear, threshold, or hormetic model (Figure 7). Study designs should include doses that most closely reflect the expected exposure levels. A critical gap that needs to be filled urgently in this context is the complete lack of data for human or environmental exposure levels of NSP. Furthermore, some knowledge about the biokinetics of NSP is required in order to estimate appropriate doses. Do specific engineered NP reach certain target sites? If so, what are the doses, dose rates, their persistence? Further, while it may be tempting to extrapolate from *in vitro* results to an *in vivo* risk assessment, it is important to keep in mind that *in vitro* tests are most useful in providing information on mechanistic processes and to elucidate mechanisms/mode of actions suggested by studies in whole animals. . A combination of *in vitro* and *in vivo* studies with relevant dose levels will be most useful in identifying the potential hazards of engineered NP, and a thorough discussion and justification of selected dose levels should be mandatory.

4. Portals of Entry and Target Tissues

Most of the toxicity research on NSP *in vivo* has been carried out in mammalian systems, with a focus on respiratory system exposures for testing the hypothesis that airborne UFP cause significant health effects. With respect to NP, other exposure routes, *via* skin and GI tract, also need to be considered as potential portals of entry. Portal of entry specific defense mechanisms protect the mammalian organism from harmful materials. However, these defenses may not always be as effective for NSP as will be discussed in this section.

4.1 The Respiratory Tract

In order to appreciate what dose the organism receives when airborne particles are inhaled, information about their deposition as well as on their subsequent fate is needed. This section will focus on the fate of inhaled nano-sized materials both within the respiratory tract itself, and translocation out of the respiratory tract. As will be pointed out, there are significant differences between NSP and larger particles regarding their behavior during deposition and clearance in the respiratory tract.

(See web-materials for additional details.)

4.1.1 Efficient Deposition of Inhaled NSP

The main mechanism for deposition of inhaled NSP in the respiratory tract is by diffusion due to displacement when they collide with air molecules. Other deposition mechanisms of importance for larger particles, such as inertial impaction, gravitational settling, and interception, do not contribute to NSP deposition, and electrostatic precipitation occurs only in cases where NSP carry significant electric charges. Figure 8 shows the fractional deposition of inhaled particles in the nasopharyngeal, tracheobronchial and alveolar regions of the human respiratory tract under conditions of nose-breathing during rest, based on a predictive mathematical model (ICRP 1994). These predictions apply to particles which are inhaled as singlet particles of a given size and not as aggregates; the latter obviously will increase particle size and change deposition site. In each of the three regions of the respiratory tract significant amounts of a certain size of NSP (1-100 nm), are deposited. For example, 90% of inhaled 1 nm particles are deposited in the nasopharyngeal compartment, only ~10% in the tracheobronchial region, and essentially none in the alveolar region. On the other hand, 5 nm particles show about equal deposition of ~30% of the

inhaled particles in all three regions; 20 nm particles have the highest deposition efficiency in the alveolar region (~50%), whereas in tracheobronchial and nasopharyngeal regions this particle size deposits with ~15% efficiency. These different deposition efficiencies should have consequences for potential effects induced by inhaled NSP of different sizes as well as for their disposition to extrapulmonary organs, as will be discussed later.

4.1.2 Disposition of NSP in the Respiratory Tract

The previous section summarized data demonstrating that inhaled NSP of different sizes can target all three regions of the respiratory tract. Several defense mechanisms exist throughout the respiratory tract aimed at keeping the mucosal surfaces free from cell debris and particles deposited by inhalation. Several reviews describe the well-known classic clearance mechanisms and pathways for deposited particles, (EPA 1996; Kreyling and Scheuch 2000; Schlesinger et al. 1997), so the following paragraphs will only briefly mention those mechanisms and point out specific differences that exist with respect to inhaled NSP.
(See web-materials for additional details.)

Once deposited, NSP – in contrast to larger-sized particles – appear to translocate readily to extrapulmonary sites and reach other target organs by different transfer routes and mechanisms. One involves transcytosis across epithelia of the respiratory tract into the interstitium and access to the blood circulation directly or *via* lymphatics resulting in distribution throughout the body. The other is a not generally recognized mechanism which appears to be distinct for NSP and which involves their uptake by sensory nerve endings embedded in airway epithelia, followed by axonal translocation to ganglionic and CNS structures.

Classical Clearance Pathways

The clearance of deposited particles in the respiratory tract is basically due to two processes (Table 3): i) physical translocation of particles by different mechanisms and (ii) chemical dissolution or leaching. Chemical dissolution is directed at biosoluble particles or components of particles that are either lipid soluble or soluble in intracellular and extracellular fluids. Solutes and soluble components can then undergo absorption and diffusion or binding to proteins and other subcellular structures, and may be eventually cleared into blood and lymphatic circulation. This mechanism of clearance for biosoluble materials can happen at any location

within the three regions of the respiratory tract, although to different degrees, depending on local extracellular and intracellular conditions (pH). In contrast, a number of diverse processes involving physical translocation of inhaled particles exist which are different in the three regions of the respiratory tract. Figure 9 summarizes these clearance processes for solid particles. As will be pointed out, some of them show significant particle size–dependent differences, making them uniquely effective for a certain particle size but very inefficient for other sizes.

The most prevalent mechanism for solid particle clearance in the alveolar region is mediated by alveolar macrophages, through phagocytosis of deposited particles. The success of macrophage-particle encounter appears to be facilitated by chemotactic attraction of alveolar macrophages to the site of particle deposition (Warheit et al. 1988). The chemotactic signal is most likely C5a, derived from activation of the complement cascade from serum proteins present on the alveolar surface (Warheit et al. 1986; Warheit and Hartsky 1993). This is followed by gradual movement of the macrophages with internalized particles towards the mucociliary escalator. The retention halftime of solid particles in the alveolar region based on this clearance mechanism is about 70 days in rats and up to 700 days in humans. The efficacy of this clearance mechanism depends highly on the efficiency of alveolar macrophages to "sense" deposited particles, move to the site of their deposition, and then phagocytize them. This process of phagocytosis of deposited particles takes place within a few hours, so that by 6-12 hours after deposition essentially all of the particles will be phagocytized by alveolar macrophages, to be cleared subsequently by the slow alveolar clearance mentioned above. However, it appears that there are significant particle size–dependent differences in the cascade of events leading to effective alveolar macrophage mediated clearance.

Figure 10 displays results of several studies in which rats had been exposed to different sized particles (for the 3 and 10 μm particles intratracheal instillation of 40 μg and 10 μg polystyrene beads were used) (Kreyling et al. 2002; Oberdörster et al. 1992b; Oberdörster et al. 2000; Semmler et al. 2004). Twenty-four hours later the lungs of the animals were lavaged repeatedly retrieving about 80% of the total macrophages as determined in earlier lavage experiments (Ferin et al. 1991). As shown in Figure 10, ~80% of 0.5, 3 and 10 μm particles could be retrieved with the macrophages, whereas only ~20% of nano-sized 15-20 nm and 80 nm particles could be lavaged with the macrophages. In effect, ~80% of the ultrafine particles were retained in the lavaged lung after exhaustive lavage, while ~20% of the larger particles > 0.5 μm

remained in the lavaged lung. This indicates that NSP were either in epithelial cells or had further translocated to the interstitium.
(See web-materials for additional details.)

Epithelial Translocation

Because of the apparent inefficiency of alveolar macrophage phagocytosis of NSP, one might expect that these particles interact instead with epithelial cells. Indeed, results from several studies show that NSP deposited in the respiratory tract readily gain access to epithelial and interstitial sites. This was also shown in studies with ultrafine PTFE fumes when shortly after a 15-min. exposure the fluorine-containing particles could be found in interstitial and submucosal sites of the conducting airways as well as in the interstitium of the lung periphery close to the pleura (Oberdörster 2000). Such interstitial translocation represents a shift in target site away from the alveolar space to the interstitium, potentially causing direct particle-induced effects there.

Indeed, a surprising finding in a study evaluating the pulmonary inflammatory response of TiO_2 particles, ranging from NP TiO_2 to pigment grade TiO_2 (12-250 nm) was that 24 hours after intratracheal instillation of different doses, higher doses induced a lower effect (Oberdörster et al. 1992a). This was explained by the additional finding that at the higher doses (expressed as particle surface area) of the nano-sized TiO_2, 50% or more had reached the pulmonary interstitium, causing a shift of the inflammatory cell response from the alveolar space to the interstitium (more information on web). The smaller particle size of 12 and 20 nm *vs*. 220 and 250 nm also means that the administered particle number was more than 3 orders of magnitude higher for the NSP, a factor that seems to be an important determinant for particle translocation across the alveolar epithelium, as are the delivered total dose and the dose rate (Ferin et al. 1992). Since interstitial translocation of fine particles across the alveolar epithelium is more prominent in larger species (dogs, non-human primates) than in rodents (Kreyling and Scheuch 2000; Nikula et al. 1997), it is reasonable to assume that the high translocation of NSP observed in rats occurs in humans as well.
(See web-materials for additional details.)

Translocation to the Circulatory System

Once the particles have reached pulmonary interstitial sites, uptake into the blood circulation in addition to lymphatic pathways can occur, a pathway that again is dependent on particle size, favoring NSP. Berry *et al.*(1977) were the first to describe translocation of NSP across the alveolar epithelium using intratracheal instillations of 30 nm gold particles in rats. They found within 30 minutes post-exposure large amounts of these particles in platelets of pulmonary capillaries; they suggested that this is an elimination pathway for inhaled particles which is of significance for transporting the finest air pollutant particles, in particular particles of tobacco smoke, to distant organs. They also hypothesized that this "might predispose to platelet aggregation with formation of microthrombi atheromatous plaques".

Since then, a number of studies with different particle types have confirmed the existence of this translocation pathway, as summarized in Table 4. Collectively, these studies indicate that particle size and surface chemistry (coating), and possibly charge, govern translocation across epithelial and endothelial cell layers. In particular, the studies summarized by Mehta (2004) and those performed by Heckel (2004)using intravenous administration of albumin-coated gold nanoparticles in rodents demonstrated receptor-mediated transcytosis (albumin binding proteins) *via* caveolae (Figure11). These 50-100 nm vesicles, first described by Simionescu et al (1975) form from indentations of the plasmalemma, and are coated with the caveolin-1 protein. Albumin, as the most abundant protein in plasma and interstitium, appears to facilitate NP endocytosis, as does lecithin, a phospholipid: Even 240 nm polystyrene particles translocated across the alveolo-capillary barrier when coated with lecithin, whereas uncoated particles did not (Kato et al. 2003). The presence of both albumin and phospholipids in alveolar epithelial lining fluid may, therefore, be important constituents for facilitated epithelial cell uptake of NSP after deposition in the alveolar space.

Rejman et al. (2004) reviewed a number of different endocytic pathways for internalization of a variety of substances, including phagocytosis, macropinocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis. They found in non-phagocytic cells *in vitro* that internalization *via* clathrin-coated pits prevailed for latex microspheres <200 nm, whereas with increasing size up to 500 nm caveolae became the predominant pathway. However, as shown in Table 4, surface coating of NSP with albumin clearly causes even the smallest particles to be internalized *via* caveolae. The presence of caveolae on cells differs, they are abundant in lung capillaries and alveolar type l cells, but not in brain capillaries (Gumbleton

2001). In the lung, during inspiratory expansion and expiratory contraction of the alveolar walls, caveolae with openings around 40 nm disappear and reappear, forming vesicles which are thought to function as transport pathways across the cells for macromolecules (Patton 1996). Knowledge from virology about cell entry of biological NSP (viruses) *via* clathrin-coated pits and caveolae mechanisms should also be considered (Smith and Helenius, 2004), and can shed light on the mechanism by which engineered NP may enter cells and interact with subcellular structures.

Evidence in humans for the translocation of inhaled NSP into the blood circulation is ambiguous, with one study showing rapid appearance in the blood and significant accumulation of label in the liver of humans inhaling ^{99}Tc-labelled 20 nm carbon particles (Nemmar et al. 2002a), while another study using the same labeled particles reported no such accumulation (Brown et al. 2002). Taken together all of the evidence from animal and human studies for alveolar translocation of NSP, it is likely that this pathway exists in humans as well; however, the extent of extrapulmonary translocation is highly dependent on particle surface characteristics/chemistry, in addition to particle size. Translocation to the blood circulation could provide a mechanism for a direct particle effect on the cardiovascular system as an explanation for epidemiological findings of cardiovascular effects associated with inhaled ambient UFP (Pekkanen et al., 2002; Wichmann et al., 2000) and for results of clinical studies showing vascular responses to inhaled ultrafine elemental carbon particles (Pietropaoli et al. 2004). In addition to direct alveolar translocation of NSP, cardiovascular effects may also be the corollary of a sequence of events starting with particle-induced alveolar inflammation initiating a systemic acute phase response with changes in blood coagulability and resulting in cardiovascular effects (Seaton et al. 1995).

Once NSP have translocated to the blood circulation, they can be distributed throughout the body. The liver is the major distribution site *via* uptake by Kupffer cells followed by the spleen as another organ of the reticuloendothelial system, although coating with PEG prevents almost completely hepatic and splenic localization so that other organs can be targeted (Akerman et al., 2002). Distribution to heart, kidney and immune-modulating organs (spleen, bone marrow) have been reported. For example, several types of NP, ranging from 10-240 nm, localized to a significant degree in bone marrow following i.v. injection into mice (Table 5). Such target specificity may be extremely valuable for drug delivery; for example, drug delivery to the CNS *via* blood-borne NP requires NP surface modifications in order to facilitate translocation across

the tight blood-brain barrier *via* specific receptors (*e.g.*, apolipoprotein coating for LDL receptor mediated endocytosis in brain capillaries) (Kreuter. 2001, 2004; Kreuter et al. 2002). Such highly desirable properties of NP must be carefully weighed against potential adverse cellular responses of targeted NP drug delivery, and a rigorous toxicological assessment is mandatory.
(See web-materials for additional details.)

Neuronal Uptake and Translocation

A translocation pathway for solid particles in the respiratory tract involving neuronal axons is apparently specific for NSP. Respective studies are summarized in Table 6. This pathway was already described more than 60 years ago, yet it has received only little or no attention by toxicologists. It is depicted in Figure 9 for the nasal and tracheobronchial regions, comprising sensory nerve endings of the olfactory and the trigeminus nerves and of an intricate network of sensory nerve endings in the tracheobronchial region. These early studies concerned a large series of studies with 30 nm polio virus intranasally instilled into chimpanzees and Rhesus monkeys (Bodian and Howe 1941; Bodian and Howe 1941; Howe and Bodian 1940). Their studies revealed that the olfactory nerve and olfactory bulbs are, indeed, portals of entry to the CNS for intranasally-instilled nano-sized polio virus particles, which could subsequently be recovered from the olfactory bulbs. The close proximity of nasal olfactory mucosa and olfactory bulb requires only a short distance to be covered by neuronal transport (Fig. 12). Bodian and Howe (Bodian and Howe 1941) determined the transport velocity of the virus in the axoplasm of axons to be 2.4 mm/hr., which is very well in agreement with neuronal transport velocities measured later by Adams and Bray (1983) for solid particles (up to 500 nm) directly microinjected into giant axons of crabs, and by deLorenzo (1970) for silver-coated colloidal gold (50 nm) in squirrel monkeys.

The de Lorenzo (1970) study demonstrated in squirrel monkeys that intranasally-instilled silver-coated colloidal gold particles (50 nm) translocated anterogradely in the axons of the olfactory nerves to the olfactory bulbs. The 50 nm gold particles even crossed synapses in the olfactory glomerulus to reach mitral cell dendrites within one hour after intranasal instillation. An interesting finding in this study — and important for potential adverse effects — was that the NSP in the olfactory bulb were no longer freely distributed in the cytoplasm but were preferentially located in mitochondria (see also section 3.3).

Newer studies indicated that this translocation pathway is also operational for inhaled NSP. Inhalation of ultrafine elemental ^{13}C particles (CMD=35 nm) resulted in a significant increase of ^{13}C in the olfactory bulb on day 1, which increased further throughout day 7 post-exposure (Oberdörster et al. 2004). Results of another inhalation study with solid nano-sized (CMD=30 nm) manganese oxide particles in rats showed after a 12-day exposure a more than 3.5-fold significant increase of Mn in the olfactory bulb, compared to only a doubling of Mn in the lung. When one nostril was occluded during a 6-hr. exposure, Mn accumulation in the olfactory bulb was restricted to the side of the open nostril only (Figure 13) (Feikert et al. 2004). This result contrasts with 15-day inhalation of larger-sized MnO_2 particles in rats (1.3 and 18 μm MMAD) where no significant increases in olfactory Mn was found (Fechter et al. 2002). This was to be expected given that the individual axons of the fila olfactoria (forming the olfactory nerve) are only 100-200 nm in diameter (De Lorenzo, 1957; Plattig, 1989

Collectively, these studies point out that the olfactory nerve pathway should also be considered a portal of entry to the CNS for humans under conditions of environmental and occupational exposures to airborne NSP. However, there are important differences between rodents and humans. The olfactory mucosa of the human nose comprises only 5% of the total nasal mucosal surface as opposed to 50% in rats – which in addition are obligatory nose breathers (Table 7). One can argue that the olfactory route may, therefore, be an important transfer route to the CNS for inhaled NSP in animals with a well-developed olfaction system, yet at the same time its importance for humans with a more rudimentary olfactory system can be questioned. However, estimates using a predictive particle deposition model and data from Table 7 show that concentrations of 20 nm translocated particles in the human olfactory bulb can, indeed, be 1.6 – 10 times greater than in rats. (Additional information on web).

Translocation into deeper brain structures may possibly occur as well, as shown in rats for soluble manganese (Gianutsos et al. 1997), but requires further confirmatory studies with respect to solid NSP. Further evidence for movement of NSP along axons and dendrites in humans is provided by knowledge accumulated by virologists who have long understood the movement of human meningitis virus through olfactory and trigeminal neurons, and, similarly, herpes virus movement up and down the trigeminal neuron to trigger outbreaks of herpes cold sores in humans (Kennedy and Chaudhuri 2002; Terasaki et al. 1997).

There are additional neuronal translocation pathways for solid NSP *via* the trigeminus nerve and tracheobronchial sensory nerves (Table 6). A study by Hunter and Dey (1998) in rats demonstrated the translocation of intranasally-instilled rhodamine-labelled microspheres (20-200 nm) to the trigeminal ganglion inside the cranium *via* uptake into the ophthalmic and maxillary branches of the trigeminus nerve which supplies sensory nerve endings throughout the nasal mucosa. Hunter and Undem (1999) in another study instilled the same microparticles intratracheally into guinea pigs; they found neuronal translocation of these solid microparticles to the ganglion nodosum in the neck area which is networked into the vagal system. This finding may be relevant for ambient UFP since it can be hypothesized that cardiovascular effects associated with ambient particles in epidemiological studies (Utell et al. 2002) are in part due to direct effects of translocated UFP on the autonomic nervous system *via* sensory nerves in the respiratory tract.

In the context of potential CNS effects of air pollution, including ambient UFP, two recent studies with exposures of mice to concentrated ambient fine and ultrafine particles should be mentioned. The authors found significant increases of TNF or decreases in dopaminergic neurons, supporting the hypothesis of ambient PM causing neurodegenerative disease (Campbell et al., 2005; Veronesi et al. 2005). A study by Calderon-Garcidueñas et al. (2002) may also point to an interesting link between air pollution and CNS effects: These authors describe significant inflammatory or neurodegenerative changes in the olfactory mucosa, olfactory bulb and cortical and subcortical brain structures in dogs from a heavily polluted area in Mexico City, whereas these changes were not seen in dogs from a little polluted rural control city. However, whether direct effects of airborne UFP are the cause of these effects remains to be determined.

Although the existence of neuronal translocation of NSP has been well established, it needs to be emphasized that size alone is only one particle parameter governing this process. Surface characteristics of NSP (chemistry, charge, shape, aggregation) are essential determinants as well, and it needs to be cautioned to assume that all NSP, when inhaled, will be distributing by the mechanism described here. It should be kept in mind though, that the unique biokinetic behavior of NSP — cellular endocytosis, transcytosis, neuronal and circulatory translocation and distribution — which makes them desirable for medical therapeutic or diagnostic applications — may be are associated with potential toxicity. For example, NP facilitated drug delivery to the CNS raises the question of the fate of NP following their translocation to specific cell types or to

subcellular structures in the brain, *e.g.*, does mitochondrial localization induce oxidative stress? How persistent is the coating or the core of the NP? A respective safety evaluation is key. (See web-materials for additional details.)

4.2 Exposure *via* GI Tract and Skin

NSP cleared from the respiratory tract *via* the mucociliary escalator can subsequently be ingested into the gastrointestinal (GI) tract. Alternatively, nanomaterials can be ingested directly, for example if contained in food or water or if used in cosmetics or as drugs or drug delivery devices. Only a few studies have investigated the uptake and disposition of nanomaterials by the GI tract, and most have shown that NSP pass through the GI tract and are eliminated rapidly. In rats dosed orally with radiolabeled functionalized C-60 fullerenes, water solubilized using PEG and albumin (18 kBq in 100 □L), 98% were cleared in the feces within 48 hours, while the rest was eliminated *via* urine, indicating some uptake into the blood circulation (Yamago et al. 1995). In contrast, in this same study, 90% of the same radiolabeled fullerenes administered i.v. (9.6 kBq, ~50□L or 14-18 kBq in 215 □L) were retained after one week, with the majority (73-80%, depending on time course) found in the liver. Studies by Kreyling (Kreyling et al., 2002, Semmler et al., 2002) using ultrafine ^{192}Ir did not show significant uptake in the GI tract, while earlier studies with larger TiO_2 particles (150-500 nm) found uptake into the blood and movement to the liver (Jani et al., 1994 and Böckmann et al. 2000). Likely there are both particle surface chemistry and particle size dependent differences in GI tract uptake.

A potentially important uptake route is through dermal exposure. The epidermis, consisting of the outer horny layer (stratum corneum), the prickle cell layer (stratum spinosum) and basal cell layer (stratum basale) forms a very tight protective layer for the underlying dermis (Fig. 14). The dermis has a rich supply of blood and tissue macrophages, lymph vessels, dendritic cells (Langerhans, also in stratum spinosum of epidermis), and five different types of sensory nerve endings. Broken skin represents a readily available portal-of-entry even for larger (0.5 – 7 μm) sized particles, as evidenced by reports about accumulation of large amounts of soil particles in inguinal lymph nodes of barefoot walking/running people; this can be associated with elephantiatic lymphedema (podoconiosis) (Corachan 1988; Price 1988). Tinkle et al. (2003) hypothesized that unbroken skin when flexed — as in wrist movements — would make the epidermis permeable for NSP. They demonstrated in a proof of concept experiment that, indeed,

flexing the skin, but not flat skin, resulted in penetration of even 1 μm fluorescent beads to the dermis. The followup question about access of particles in the dermis to the circulation is answered by the aforementioned reports of podoconiosis, *i.e.*, uptake into the lymphatic system and regional lymph nodes. Subsequent translocation of NSP beyond lymph nodes to the blood circulation is likely to occur as well, as shown in studies with small asbestos fibers (Oberdörster et al. 1988).

Newer studies by Kim et al. (2004) in mice and pigs with intradermally-injected near infrared quantum dots confirmed that NP, once in the dermis, will localize to regional lymph nodes, which makes these particles very useful for *in vivo* imaging. Likely transport mechanisms to the lymph nodes are skin macrophages and dendritic (Langerhans) cells (Sato et al. 1998; Ohl et al. 2004); this raises a question about potential modulation of immune responses, following interaction of these NP containing macrophages and dendritic cells with T-lymphocytes. For example, Chen et al. (1998) were able to raise antibodies in mice specific for C_{60} after *i.p.* injections of C_{60} conjugated to thyroglobulin and serum albumin. Clearly, research is needed to determine whether and under what conditions NP can be recognized by the immune system, following any route of uptake into the organism.

Another question relates to the potential of sensory skin nerves to take up and translocate NP: Given that this mechanism has been demonstrated for the nasal and tracheobronchial regions of the respiratory tract, how likely is this to occur in the dermis layer of the skin with its dense supply of different types of sensory nerves? It may be conceivable, considering data on neuronal uptake and translocation of NSP after intramuscular injection. For example, nano-sized ferritin and iron-dextran, after injection into the tongue of mice, labeled the neurons of the hypoglossal nuclei; and injection of both of these NSP into facial muscles of mice also resulted in synaptic uptake; cationized ferritin was also detected in cell bodies of facial neurons indicating that electrical charge is of importance for incorporation into axons and axonal transport (Arvidson 1994; Malmgren et al. 1978; Olsson and Kristensson 1981). Other studies using intra-muscular injection of ferritin (~112 nm), iron-dextran (11 or 21 nm) and gold protein (20-25 nm) nanoparticles also showed rapid penetration through the basal lamina into the synaptic clef of the neuromuscular junction, but this was restricted only to the smaller nanoparticles, implying that there may be a size-dependent penetration of the basal lamina with a threshold somewhere between 10 and 20 nm (Oldfors and Fardeau 1983).

Neuronal transport of NSP along sensory skin nerves is well established for herpes virus. After passing through the skin — especially broken skin — they are transported retrogradely along dendrites of sensory neurons to the dorsal root ganglion, remain dormant there until a stress situation triggers anterograde translocation along the dendrites back to the skin (Kennedy and Chaudhuri 2002; Terasaki et al. 1997). Future studies need to determine as to whether and to what degree such translocation along sensory skin neurons also occurs with NP penetrating the epidermis.

5. Risk Assessment

The lack of toxicology data on engineered NP does not allow for adequate risk assessment. Because of this, some may even believe engineered NP so risky that they call for a precautionary halt in NP-related research. However, the precautionary principle should not be used to stop research related to nanotechnology and NP. Instead, we should strive for a sound balance between further development of nanotechnology and the necessary research to identify potential hazards in order to develop a scientifically defensible database for the purpose of risk assessment. To be able to do this, a basic knowledge about mammalian and eco-toxicological profiles of NP is necessary, rather than attempting to assess NP risks based on some popular science fiction literature. Most importantly, sufficient resources should be allocated by governmental agencies and industries to be able to perform a scientifically based risk assessment and then establish justifiable procedures for risk management. The data needed for this risk assessment should be determined *a priori* so that limited resources can be used efficiently to develop useful and well-planned studies.

At this point, governmental regulation is not possible given the lack of needed information on which to base such regulations. However, academia and industry and regulatory governmental agencies should seriously consider the view that engineered nanoparticles have new and unique biological properties and that the potential risks of engineered NP are not the same as those of the bulk material of the same chemistry. Assigning a unique identifier to nano-sized materials would indicate that the toxicology profile of the material in question may not be the same as the bulk material. Toxicological tests and the resulting data base would provide information for MSDS sheets for NP as well as a basis for potential NP risk assessments and risk management. Obviously, this approach may not be appropriate for all NP, for example, when embedded in a

matrix, and the feasibility of this proposed strategy needs to be thoroughly discussed and considered. For discussing this, and for developing and deciding upon a reasonable battery of tests for toxicological profiling, it would be very useful to convene international multidisciplinary workshops of experts from industry, academia, and regulatory agencies (including material scientists, chemists, chemical engineers, toxicologists, physicians, regulators, statisticians, and others) to establish a NP classification scheme and testing guidelines. A multidisciplinary and multi-national collaborative team approach is critical. Respective efforts have been initiated nationally by the American Standards Institute (ANSI 2004) and internationally by the International Council on Nanotechnology (ICON 2004) as well as the International Organization for Standardization.

As many regulatory agencies do not consider a nanotechnologically manufactured substance different from the conventional substance, the manufacture and use of nanotechnology products are currently not specifically regulated. Typically, nano-sized substances are treated as variations of the technical material or existing formulation and thus do not require a separate registration. A main reason for producing nano-sized form of a registered substance, however, is that conversion of a substance to a nanoparticle imparts new properties to the substance (*e.g.*, enhanced mechanical, electrical, optical, catalytic, biological activity). Thus, as stated earlier, while the toxicology of the base material may be well defined, the toxicity of the nanotechnology form of the substance may be dramatically different from its parent form. As a result, new toxicology data on the nano-size form of a substance is likely to result in a different hazard assessment for the NP. Figure 15 depicts a Risk Assessment/Risk Management paradigm pointing out different steps and data required for this process.

As described in the previous sections, the difference in toxicological profile of NP compared to its parent form is not only due to its intrinsic chemical properties, but to a large degree to differing kinetics *in vivo*. While larger particles may not enter the CNS, the potential exists for inhaled nano-sized particles to be translocated to the CNS *via* the axons of sensory neurons in the upper respiratory tract. Furthermore, while the toxicity per unit mass of a particular substance may vary depending on the nano *vs*. large form, it will be important to take into account not only new biological activities, but also potential new target organs and routes of exposure. To what degree does the nano-form of a substance have enhanced dermal penetration, or increased systemic uptake *via* the lung or GI-tract? What determines how many nanoparticles

that enter the systemic circulation distribute throughout the body, reach the bone marrow, cross the blood brain barrier, cross the placenta, and affect the developing offspring, or sequester effectively in the liver? Do nanoparticles released into the environment affect species that are important in food chain dynamics? What are the long-term consequences of exposure to nanoparticles? Changes in toxicity profile and new target organs can be expected, and it will then be necessary to establish new risk assessments for nanoparticles in addition to the bulk material. Currently there exists a paucity of data to effectively address these questions but it will be important to determine whether there exist common modes of action/behavior of NP to establish baseline assumptions for use in risk assessments.

The use of nanotechnology products will likely increase dramatically over the next decade. In fact, nanomaterials are already being used in applications ranging from burn and wound dressings to dental-bonding agents to sunscreens and cosmetics to fuel cells, tires, optics, clothing, and electronics. While currently there exists little public awareness of nanotechnology in everyday life (*e.g.*, stain-free clothing), it would be prudent to examine and address environmental and human health concerns before the widespread adoption of nanotechnology. Both the societal benefits and potential risks of nanotechnology should be evaluated and clearly communicated to the general public and regulators. This type of open communication and risk/benefit evaluation will avoid the pitfalls encountered with genetically modified organisms recently experienced in the field of biotechnology. In that instance, the benefits of the emerging field of biotechnology were not communicated effectively before the introduction of the technology. As the public's awareness of this new technology grew, regulators and producers of biotechnology failed to effectively acknowledge public concerns that genetically modified organisms could adversely affect ecosystem balance. As a result, the public support of genetically modified organisms, particularly in the EU, is low. For nanomaterial producers it will be important to demonstrate that what they may perceive as a new and potentially harmless form of a familiar material has, indeed, an acceptable risk profile. If such proactive steps are not taken, nanomaterials may be regarded as dangerous by the public and regulators, which could lead to inappropriate categorization and unnecessarily burdensome regulations. Such action (or inaction on the side of producers), in turn, could result in significant barriers to commercialization and the widespread acceptance of otherwise useful nanotechnology materials.

6. Summary & Outlook

Research on ambient UFP has laid the foundation for the emerging field of nano-toxicology, with the goal to study the biokinetics and the potential of engineered nanomaterials (particles, tubes, shells, quantum dots, *etc*.) to cause adverse effects. Major differences between ambient UFP and NP are the polydisperse nature of the former *vs*. the monodisperse size of the latter; and particle morphology, oftentimes a branched structure from combustion *vs*. spherical form of NP, although other shapes (tubes, wires, rings, planes) are also manufactured. In addition, combustion derived volatile organic compounds and inorganic constituents (*e.g*., metals, nitrates, sulfates) of different solubilities on UFP predict differences in the toxicological profile between UFP and NP. However, as far as the insoluble particle is concerned, concepts of NSP kinetics, including cell interactions, will most likely be the same for UFP and NP (Figure 16).

The introduction of nanostructured materials for biomedical and electronics applications opens tremendous opportunities for biomedical applications as therapeutic and diagnostic tools as well as in the fields of engineering, electronics, optics, consumer products, alternative energy, soil/water remediation and others. However, very little is known yet about their potential to cause adverse effects or humoral immune responses once they are introduced into the organism — unintentionally or intentionally. Nano-medicine products will be well-tested prior to introduction into the marketplace. However, for the manufacturers of the majority of current nano-tech products, regulations requiring nanomaterial-specific data on toxicity prior to introduction into the marketplace are an evolving area and presently under discussion (Bergeson and Auerbach, 2004; Foresight and Governance Project, 2003). During a product's Life Cycle (manufacture, use, disposal), it is probable that nanomaterials will enter the environment, and currently there is no unified plan to examine ecotoxicological effects of NP. In addition, the stability of coatings and covalent surface modifications need to be determined both in ecological settings and *in vivo*. (*Additional information on web*).

Results of older biokinetic studies and some new toxicology studies with NSP (mostly ambient UFP) can be viewed as the basis for the expanding field of nanotoxicology. These studies showed that the greater surface area per mass renders NSP more active biologically than larger sized particles of the same chemistry, and that particle surface area and number appear to be better predictors for NSP-induced inflammatory and oxidative stress responses. The following emerging concepts of nanotoxicology can be identified from these studies:

- The biokinetics of nano-sized particles are different from larger particles:
  - when inhaled:
    they are efficiently deposited in all regions of the respiratory tract;
    they evade specific defense mechanisms;
    they can translocate out of the respiratory tract via different pathways (endocytosis and transcytosis)
  - when in contact with skin:
    there is evidence of penetration to the dermis;
    they translocate *via* lymph to regional lymph nodes;
    a possible uptake into sensory nerves needs to be investigated
  - when ingested:
    there appears to be little uptake into the organism, mostly excreted *via* feces
  - when in blood circulation:
    they can distribute throughout the organism,
    they are taken up into liver, spleen, bone marrow, heart, and other organs
  - in general, translocation rates are largely unknown, they are probably very low but are likely to change in a compromised/diseased state
- The biological activity and biokinetics are dependent on many parameters:
  - size; shape; chemistry; crystallinity;
  - surface properties (area, porosity, charge, surface modifications, weathering of coating)
  - agglomeration state; biopersistence; dose
- These parameters are likely to modify responses and cell interactions such as:
  - a greater inflammatory potential than larger particles per given mass
  - translocation across epithelia from portal-of-entry to other organs
  - translocation along axons and dendrites of neurons
  - induction of oxidative stress
  - pro-oxidant and antioxidant activity of NSP in environmentally-relevant species
  - binding to proteins, receptors
  - localization in mitochondria

The principles of cellular and organismal interactions that have been discussed in this manuscript, should be applicable for both ambient UFP and NP, even if the latter are coated with a biocompatible material. Knowledge about the biopersistence of this coating is as essential as is knowledge about the bioavailability of the core material that could have intrinsic toxic properties, *e.g.*, semiconductor metal compounds in sub 10 nm quantum dots consisting of cadmium and lead compounds. The very small size of these materials makes them available to the same translocation processes described here for polydisperse NSP, possibly even in a more efficient way because of their uniform size. When studying biological/toxicological effects, new processes of interactions with subcellular structures, *e.g.*, microtubuli, mitochondria, will likely be discovered. The diversity of engineered nanomaterials and of the potential effects represents major challenges and research needs for nanotoxicology, including also the need for assessing human exposure during manufacture and usage. The goal to exploit positive aspects of engineered nanomaterials and avoid potential toxic effects can best be achieved through a multidisciplinary team effort involving researchers in toxicology, materials science, medicine, molecular biology, bioinformatics and their subspecialties.

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8. TABLES:

Table 1: Ultrafine/Nano Particles (<100 nm): Natural and anthropogenic sources.

Natural	Anthropogenic	
	Unintentional	Intentional
Gas to particle conversions	Internal combustion engines	Engineered nanoparticles:
Forest fires	Power plants	(*controlled size and shape,*
Volcanoes (hot lava)	Incinerators	*designed for functionality)*
Viruses	Airplane jets	*metals, semiconductors, metal oxides*
Biogenic magnetite:	Metal fumes	*carbon, polymers*
magnetotactic bacteria	*(smelting, welding, etc.)*	*nano-spheres, -wires,*
protoctists, mollusks,	Polymer fumes	*-needles, -tubes, -shells,*
arthropods, fish, birds	Other fumes	*-rings, -platelets*;
human brain, meteorite?	Heated surfaces	*untreated, coated*
Ferritin (12.5 nm)	Frying, broiling, grilling	*(nanotechnology applied to many*
Microparticles *(<100 nm)*	Electric motors	*products: cosmetics, medical, fabrics,*
(activated cells)		*electronics, optics, displays, etc.)*

Portals of Entry

Respiratory Tract	***GI-tract***	***Skin***	***Injection***
Inhalation	*Ingestion*	*Dermal*	*Blood Circulation*

Table 2: Particle Number and Particle Surface Area per 10 μg/m^3 Airborne Particles.

Particle Diameter μm	Particle Number cm^{-3}	Particle Surface Area μm^2/cm^3
5	153,000,000	12,000
20	2,400,000	3,016
250	1,200	240
5,000	0.15	12

Table 3: Clearance Mechanisms for Inhaled Solid Particles in the Respiratory Tract

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Physical Clearance Processes (*Translocation*)

- mucociliary movement (*nasal; tracheobronchial*)
- macrophage phagocytosis (*tracheobronchial; alveolar*)
- epithelial endocytosis (*nasal, tracheobronchial; alveolar*)
- interstitial translocation (*tracheobronchial; alveolar*)
- lymphatic drainage (*tracheobronchial; alveolar*)
- blood circulation (*alveolar*)
- sensory neurons (*nasal; tracheobronchial*)

Chemical Clearance Processes

- dissolution
- leaching
- protein binding

} *all 3 regions*

Table 4: Particle Size and Surface Chemistry-related Alveolar-Capillary Translocation

Particle size (nm)	Type	Translocation	Localization/Effect	Reference
5-20 nm	Gold, albumin coated	yes	via caveolae	Mehta et al., 2004
8 nm	Gold, albumin coated	yes	via "vesicles"	Konig et al., 1993
8 nm	Gold, albumin coated	yes	via caveolae	Heckle et al., 2004
18 nm	Iridium	yes[a]	extrapulm. org.	Kreyling et al., 2002
30 nm	Gold	yes	platelet?	Berry et al., 1977
35 nm	Carbon	yes	liver	Oberdörster et al., 2002
60 nm	Polystyrene, [b]	yes	thrombus, early	Nemmar et al., 2002b Silva et al., 2005
60 nm	Polystyrene	?	no thrombus	Nemmar et al., 2002b
80 nm	Iridium	yes[a]	extrapulm. org.	Kreyling et al., 2002
240 nm	Polystyrene, lecithin	yes	monocyte	Kato et al., 2003
240 nm	Polystyrene, uncoated	no		Kato et al., 2003
400 nm	Polystyrene	no	thrombus, late	Nemmar et al., 2004

a = minimal; b = indirect evidence

Surface coating (chemistry) charge, size govern translocation

Table 5: Translocation of Nano-sized Particles in the Blood Circulation to Bone Marrow in Mice.

Particle Size	Type	Finding	Reference
~10 nm	PEG-quantum dots	Fast appearance of QDs in liver, spleen, lymph nodes and bone marrow (mouse)	Ballou et al., 2004
<220 nm	Metallo-fullerene	Highest accumulation in bone marrow after liver; continued increase in bone marrow but decrease in liver (mouse)	Cagle et al., 1999
90 – 250 nm	HAS coated polylactic acid nanoparticles	Significant accumulation in bone marrow, second to liver (rat)	Bazile et al., 1992
240 nm	Polystyrene (non biodegradable) polylisohexylcyon-acrylate (biodegradable)	Rapid passage through endothelium in bone marrow, uptake by phagocytizing cells in tissue (mouse)	Gibaud et al., 1996 1998 1994

Table 6: Studies of Neuronal Translocation of UFP from Respiratory Tract

1941:	*Bodian and Howe*: **Olfactory** axonal transport of Polio-virus (30 nm) after intranasal instillation in chimpanzee. Transport velocity: 2.4 mm/h
1970:	*de Lorenzo*: **Olfactory** axonal transport of 50 nm silver-coated gold after intranasal instillation in squirrel monkey. Transport velocity: 2.5 mm/h
1998:	*Hunter and Dey*: Retrograde tracing of **trigeminal** neurons from nasal epithelium with microspheres
1999:	*Hunter and Undem*: Rhodamine-labelled microspheres (20-200 nm) translocation via sensory nerves of **TB region** to ganglion nodosum in hamster after intratracheal instillation
2004a:	*Oberdörster et al.*: ^{13}C particles (CMD ~36 nm) in **olfactory** bulb after whole-body inhalation exposure in rats

Table 7: Rat vs. Human Nasal and Olfactory Parameters.

	Rat	Human
Breathing mode	obligatory nose	nasal/oro-nasal
Area of nasal mucosa	~16 cm^3	~105 cm^2
Area of olfactory mucosa (% of total mucosa)	~8 cm^3 (50)	~5.25 cm^2 (5)
% of nasal airflow going to olfactory mucosa	~15	~10
Weight of olfactory bulb	~85 ng	~168 ng

Based on Keyhani et al., 1997; Kimbell et al., 1997; Turetsky et al., 2003

9. FIGURE LEGENDS

Figure 1: Idealized size distribution of traffic-related particulate matter (EPA, 2004). The four polydisperse modes of traffic-related ambient particulate matter span approximately 4 orders of magnitude from below 1 nm to above 10 μm. Nucleation and Aitken mode particles are defined as ultrafine particles (<~100 nm). Source-dependent chemical composition is not well controlled and varies considerably. In contrast engineered nanoparticles (1-100 nm) have well controlled chemistry and are generally monodispersed.

Figure 2: Surface molecules as function of particle size. Surface molecules increase exponentially when particle size decreases below 100 nm, reflecting the importance of surface area for increased chemical and biological activity of NSP. The increased biological activity can either be positive and desirable (*e.g.*, antioxidant activity, carrier capacity for therapeutics, penetration of cellular barriers) or negative and undesirable (*e.g.,* toxicity, induction of oxidative stress or of cellular dysfunction) or also a mix of both. (*Figure courtesy of Dr. Fissan, personal communication*)

Figure 3: Hypothetical cellular interaction of NSP *(adapted from Donaldson and Tran (2002).* Inflammation and oxidative stress can be mediated by several primary pathways: 1) Particle surface causes oxidative stress resulting in increased intracellular calcium and gene activation, 2) Transition metals released from particles result in oxidative stress, increased intracellular calcium, and gene activation, 3) Cell surface receptors are activated by transition metals released from particles, resulting in subsequent gene activation, 4) Intracellular distribution of NSP to mitochondria generates oxidative stress.

Figure 4: Percent of neutrophils in lung lavage of rats and mice as indicators of inflammation 24 hrs. after intratracheal instillation of different mass doses of 20 nm and 250 nm

TiO_2 particles in rats and mice. (a) The steeper dose–response of nano-sized TiO_2 is obvious when dose is expressed as mass. (b) The same dose–response relationship but with dose expressed as particle surface area, indicating that particle surface area seems to be a more appropriate dosemetric for comparing effects of different sized particles provided they are of the same chemical structure (anatase TiO_2 in this case). (■ 20 nm TiO2; ▲ 250 nm TiO2, ● saline control)

Figure 5: Routes of exposure, uptake, distribution and degradation of NSP in the Environment. Solid lines indicate routes which have been demonstrated in the laboratory or field; or which are currently in use (remediation). Italics indicate possible degradation routes, while blue lettering indicates possible sinks and sources of NSP.

Figure 6: Engineered NP have been shown to release oxyradicals (pictured here is the mechanism of C_{60} as determined by Yamakoshi et al. (2003), which can interact with the antioxidant defense system. In addition to fullerenes, metals such as Cd, Fe, or Ni quantum dots, or Fe from SWNT manufacturing, could also act in Fenton-type reactions. Abbreviations: SOD = superoxide dismutase; GSH = reduced glutathione; GSSG = oxidized glutathione; GPx = glutathione peroxidase; R = any organic molecule.

Figure 7: Some basic shapes of exposure-response or dose-response relationships. Prerequisites for establishing these relationships for NSP from *in vitro* or *in vivo* studies include a sufficient number of data points, *i.e.*, over a wide range of exposure concentrations or doses; knowledge about exposure levels; and information about correlation of exposure with doses at the organismal or cellular level (an exposure is not a dose!). Dose-response curves of different shapes can be extrapolated when only response data at high dose levels (indicated by dashed oval) are available. Lack of data in the low – oftentimes the most relevant – dose range can result in severe misinterpretation if a threshold or even a hormetic response is present. Consideration also needs to be given to the likelihood that the shape or slope of exposure-dose-response relationships change for susceptible parts of the population.

Figure 8: Predicted fractional deposition of inhaled particles in the nasopharyngeal, tracheobronchial and alveolar region of the human respiratory tract during nose-breathing (based on data from ICRP (1994). *(Drawing courtesy of J. Harkema)*

Figure 9: Pathways of particle clearance (disposition) within and out of the respiratory tract. There are significant differences between nano-sized and larger particles for some of these pathways (see text). *(Drawing courtesy of J. Harkema).*

Figure 10: *In vivo* retention of inhaled nano-sized and larger particles in alveolar macrophages (left side) and in exhaustively lavaged lungs (epithelial and interstitial retention, right side) 24 hrs. post-exposure. The alveolar macrophage is a most important defense mechanism in the alveolar region for fine and coarse particles, yet inhaled singlet NSP are not efficiently phagocytized by alveolar macrophages.

Figure 11: Different forms of caveolae and cellular tight junctions function as translocation mechanisms across cell layers. Depending on particle surface chemistry, NSP have been shown to transcytose across alveolar type I epithelial cells and capillary endothelial cells (Table 4), but not *via* cellular tight junctions. However, in a compromised or disease state (*e.g.*, endotoxin exposure) translocation across widened tight junction occurs as well (Heckel et al., 2004). This indicates that assessing potential effects of NSP in the compromised state is an important component of Nanotoxicology.

Figure 12: Close proximity of olfactory mucosa to olfactory bulb of the CNS. Inhaled NSP, especially below 10 nm, deposit efficiently on the olfactory mucosa by diffusion, similar to airborne "smell" molecules which deposit in this area of olfactory dendritic cilia. Subsequent uptake and translocation of solid NSP along axons of the olfactory nerve has been demonstrated in non-human primates and rodents. Surface chemistry of the particles may influence their neuronal translocation. (*Figure used with permission from McGraw-Hill*)

Figure 13: Occlusion of the right nostril of rats during 6 hr. inhalation of nano-sized Mn-oxide particles (~30 nm CMD, ~450 $\mu g/m^3$) resulted in accumulation of Mn only in the left olfactory bulb only at 24 hrs. after dosing (Feikert et al., 2004).

Figure 14: The epidermis represents a tight barrier against NSP penetration. Quantitatively, dermal translocation will, therefore, be minimal or non-existent under normal conditions but increases in areas of skin flexing (Tinkle et al., 2003) and broken skin. Once in the dermis, lymphatic uptake is a major translocation route, likely facilitated by uptake in dendritic cells (epidermis) and macrophages; other potential pathways may include the dense networks of blood circulation and sensory nerves in the dermis. *(Figure adapted from: http/www.essentialdayspa.com/Skin_Anatomy_and_Physiology.htm)*

Figure 15: Risk assessment *(NRC 1983)* and risk management paradigm for engineered nanoparticles (NP). The four steps of risk assessment require answers to the questions: Do NP have adverse effects? What are the dose–response relationships? What are occupational/environmental levels in different media? What is the calculated risk? Once a risk is determined, risk management decision can be established, including exposure standards and regulations and efforts for effective risk communication.

Figure 16: Biokinetics of NSP. While many uptake and translocation routes have been demonstrated, others still are hypothetical and need to be investigated. Largely unknown are translocation rates as well as accumulation and retention in critical target sites and their underlying mechanisms. These as well as potential adverse effects will be largely dependent on physicochemical characteristics of the surface and core of NSP. Both qualitative and quantitative changes in NSP biokinetics in a diseased or compromised organism need also to be considered.

Figure 1

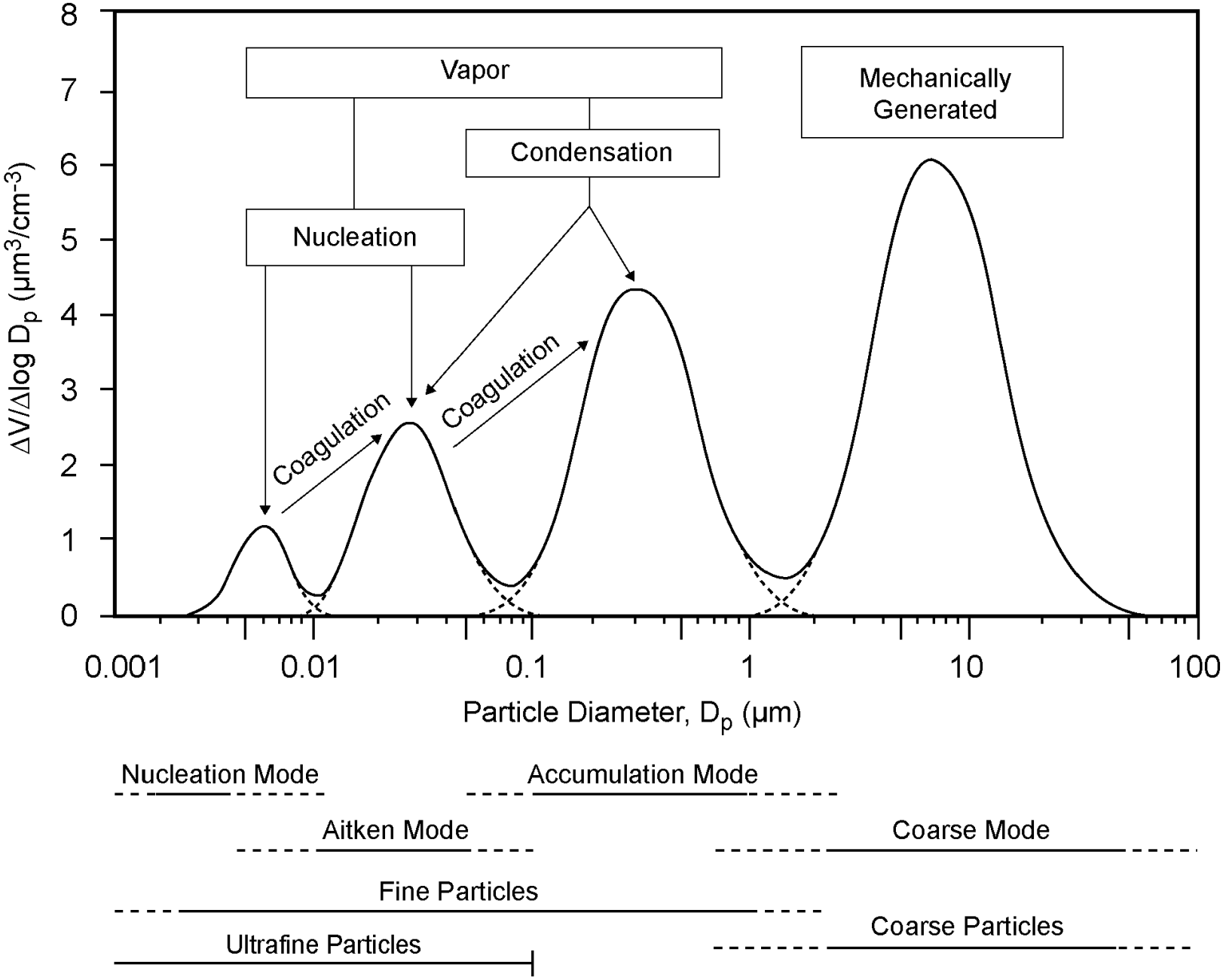

Vapor
Condensation
Nucleation
Mechanically Generated
Coagulation
Coagulation
ΔV/Δlog Dp (μm3/cm-3)
Particle Diameter, Dp (μm)
0.001
0.01
0.1
1
10
100
Nucleation Mode
Accumulation Mode
Aitken Mode
Coarse Mode
Fine Particles
Coarse Particles
Ultrafine Particles

Figure 2

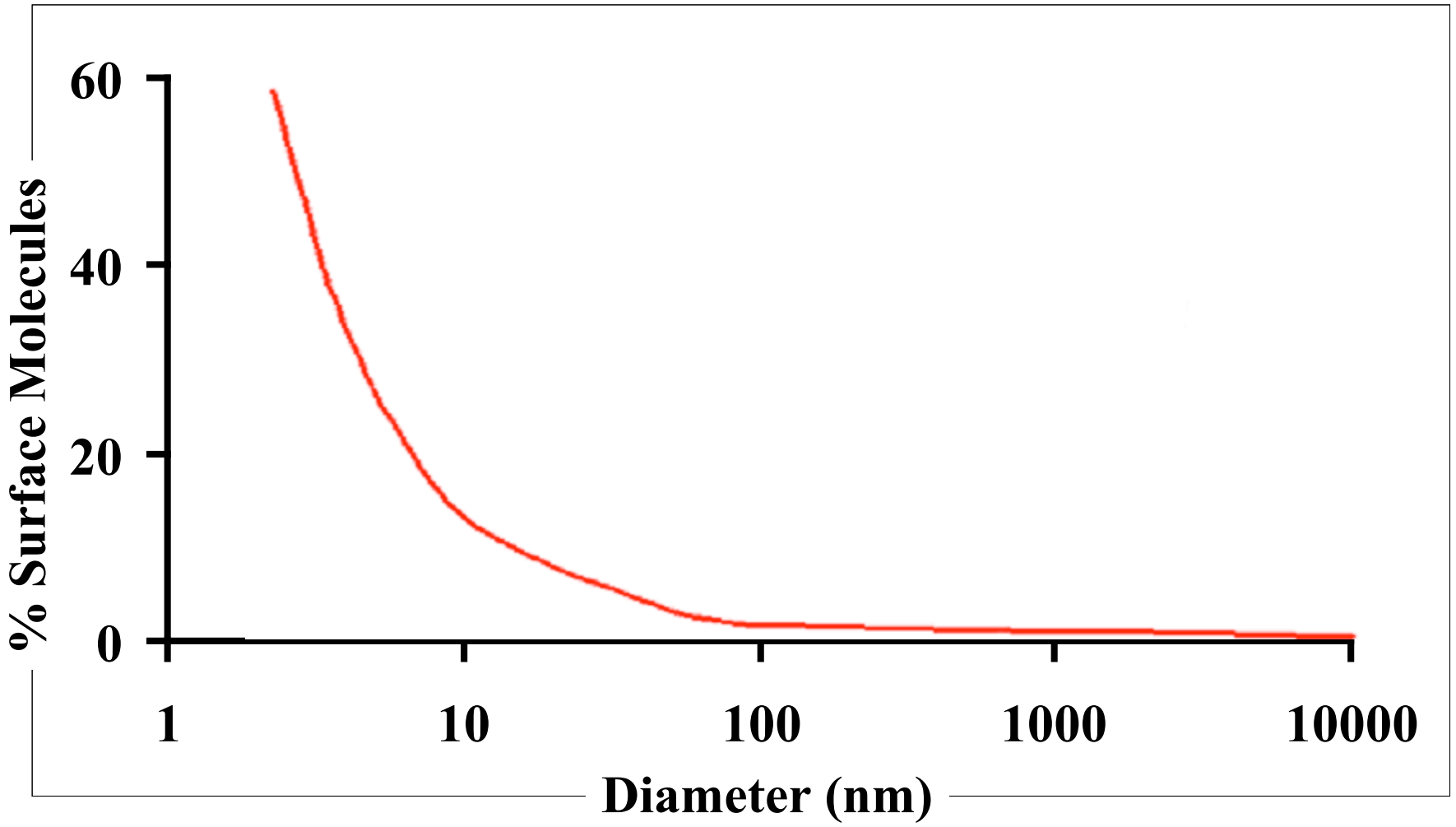


Figure 3

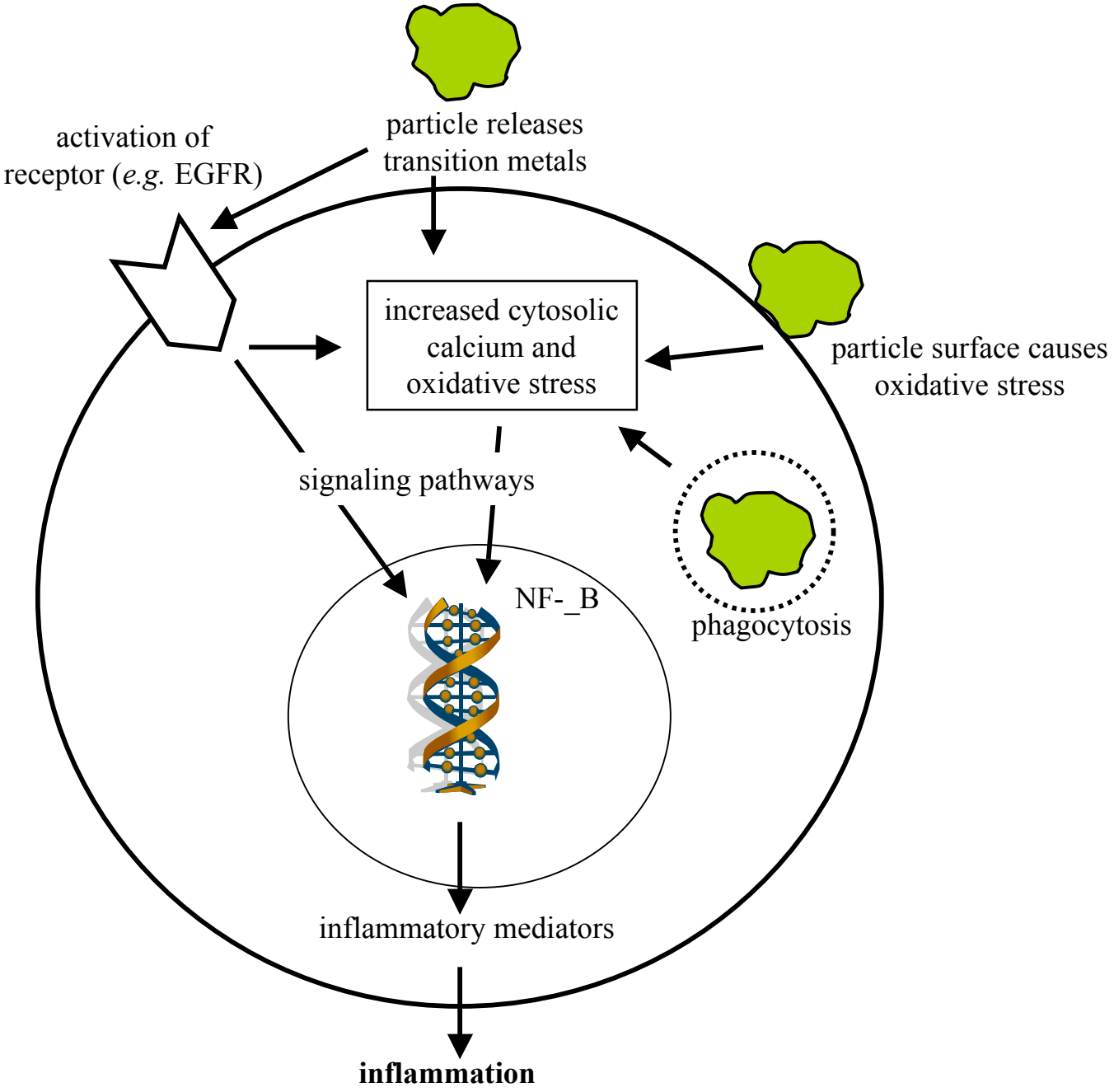

particle releases
transition metals
activation of
receptor (*e.g.* EGFR)
increased cytosolic
calcium and
oxidative stress
particle surface causes
oxidative stress
signaling pathways
NF-_B
phagocytosis
inflammatory mediators
inflammation

Figure 4

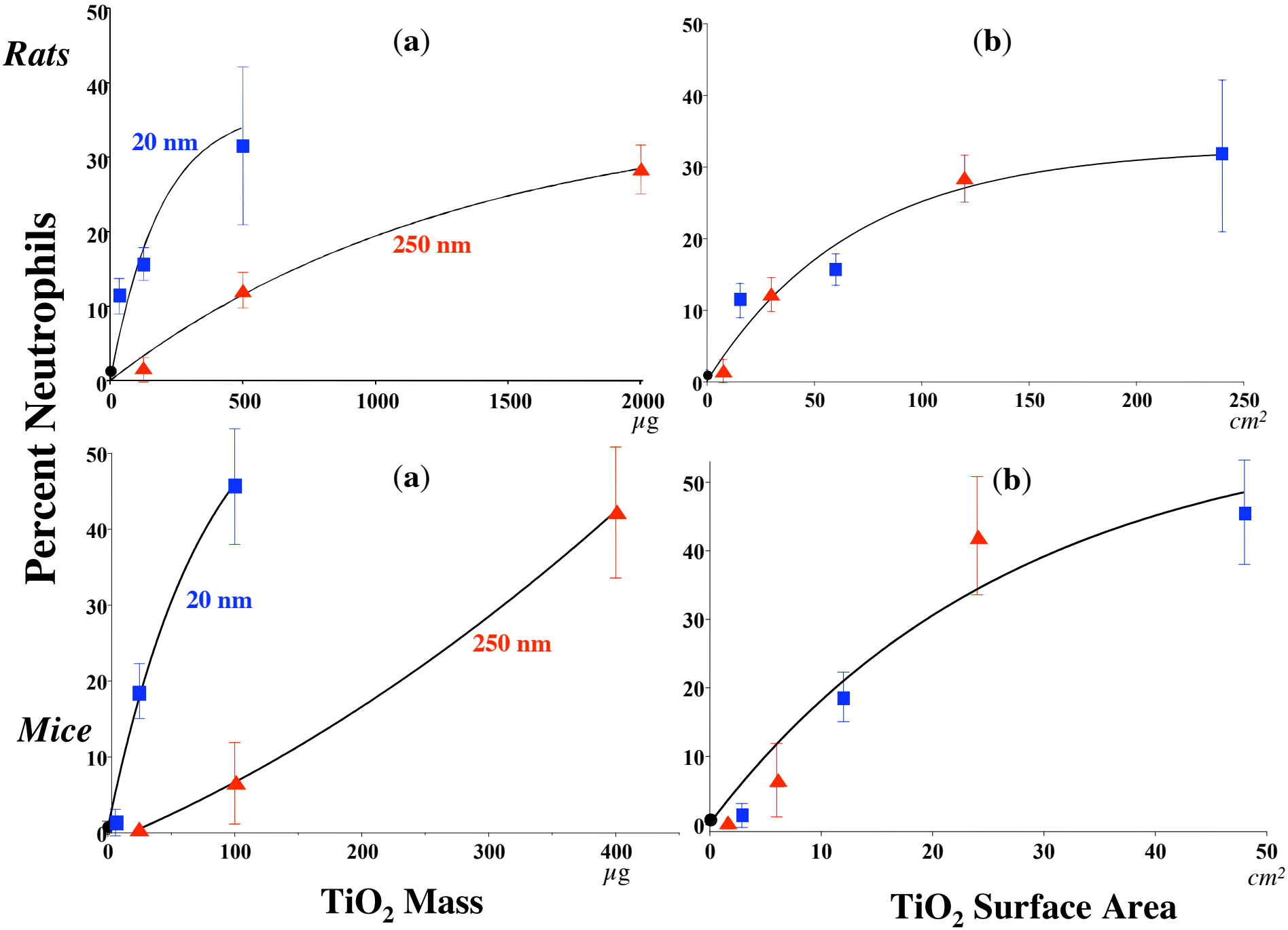
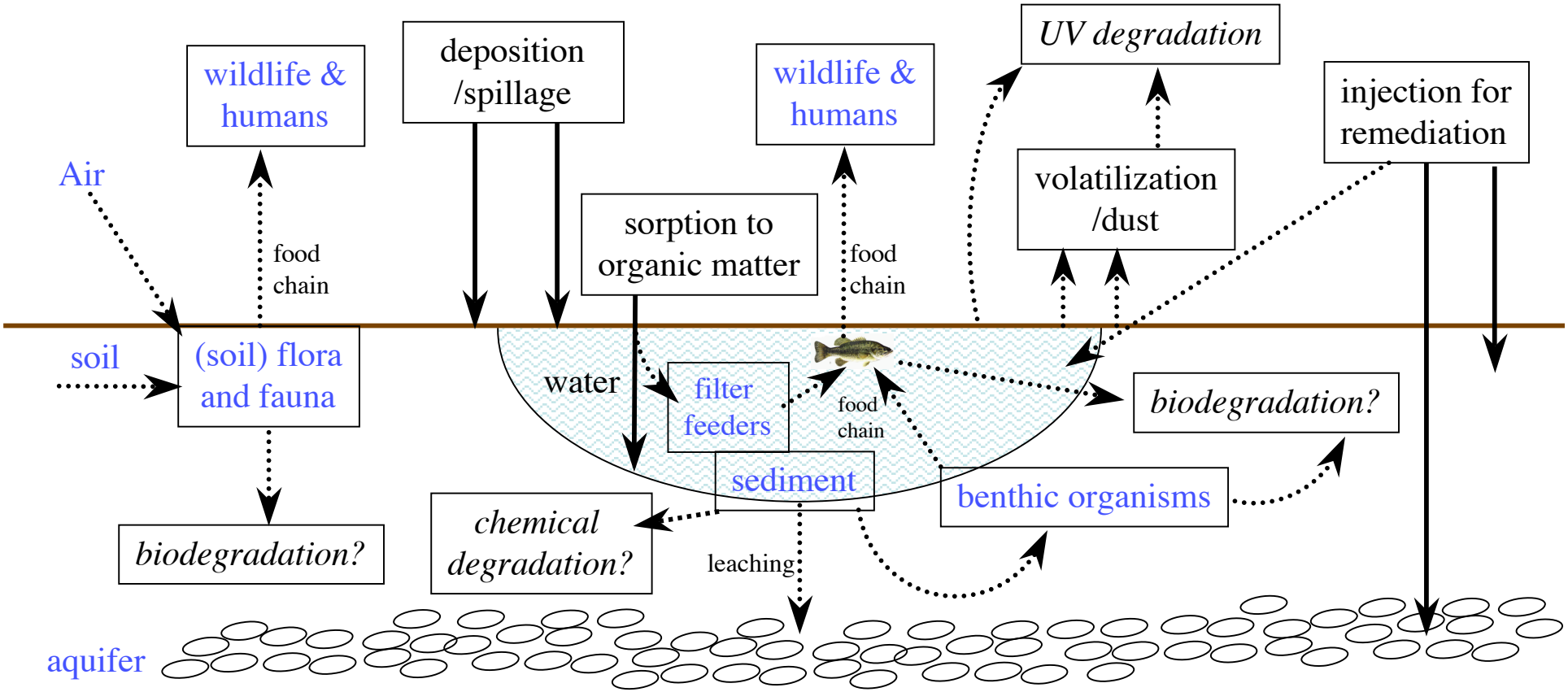
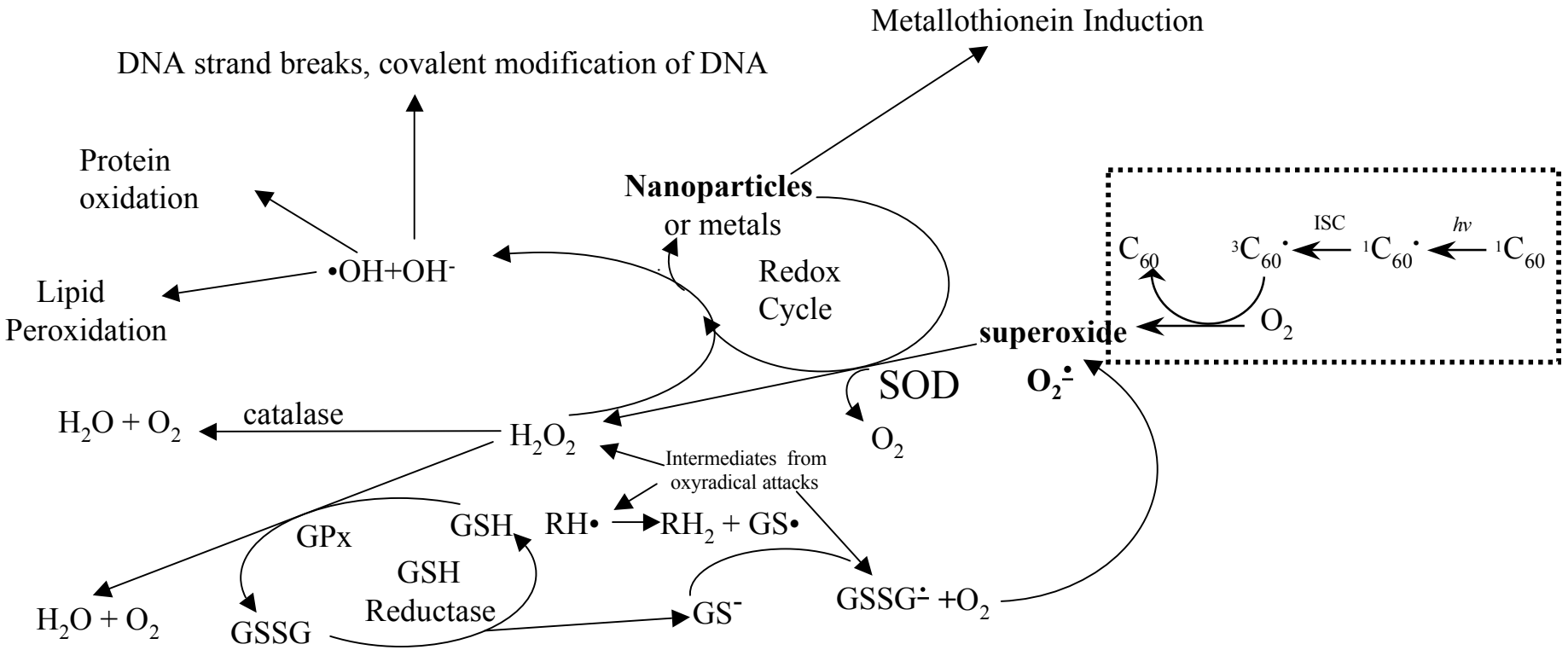

Rats
Mice
Percent Neutrophils
(a)
(b)
20 nm
250 nm
μg
cm²
TiO₂ Mass
TiO₂ Surface Area

Figure 5



Legend: Routes of exposure, uptake, distribution and degradation of NSP in the Environment. Solid lines indicate routes which have been demonstrated in the laboratory or field; or which are currently in use (remediation). Italics indicate possible degradation routes, while blue lettering indicates possible sinks and sources of NSP.

Figure 6



not shown: phase II biotransformation, ascorbic acid, vitamin E, beta carotene etc.

Figure 7

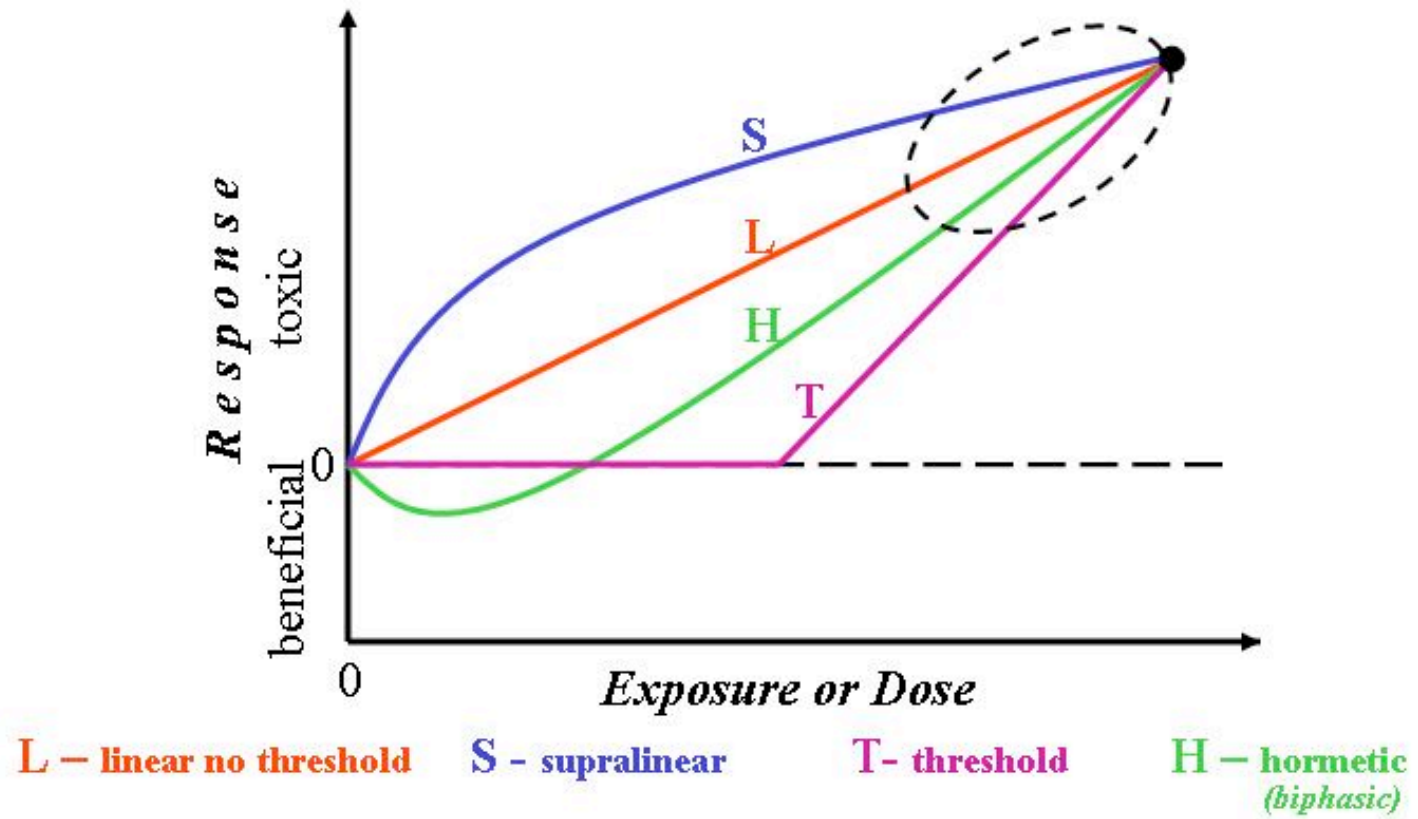
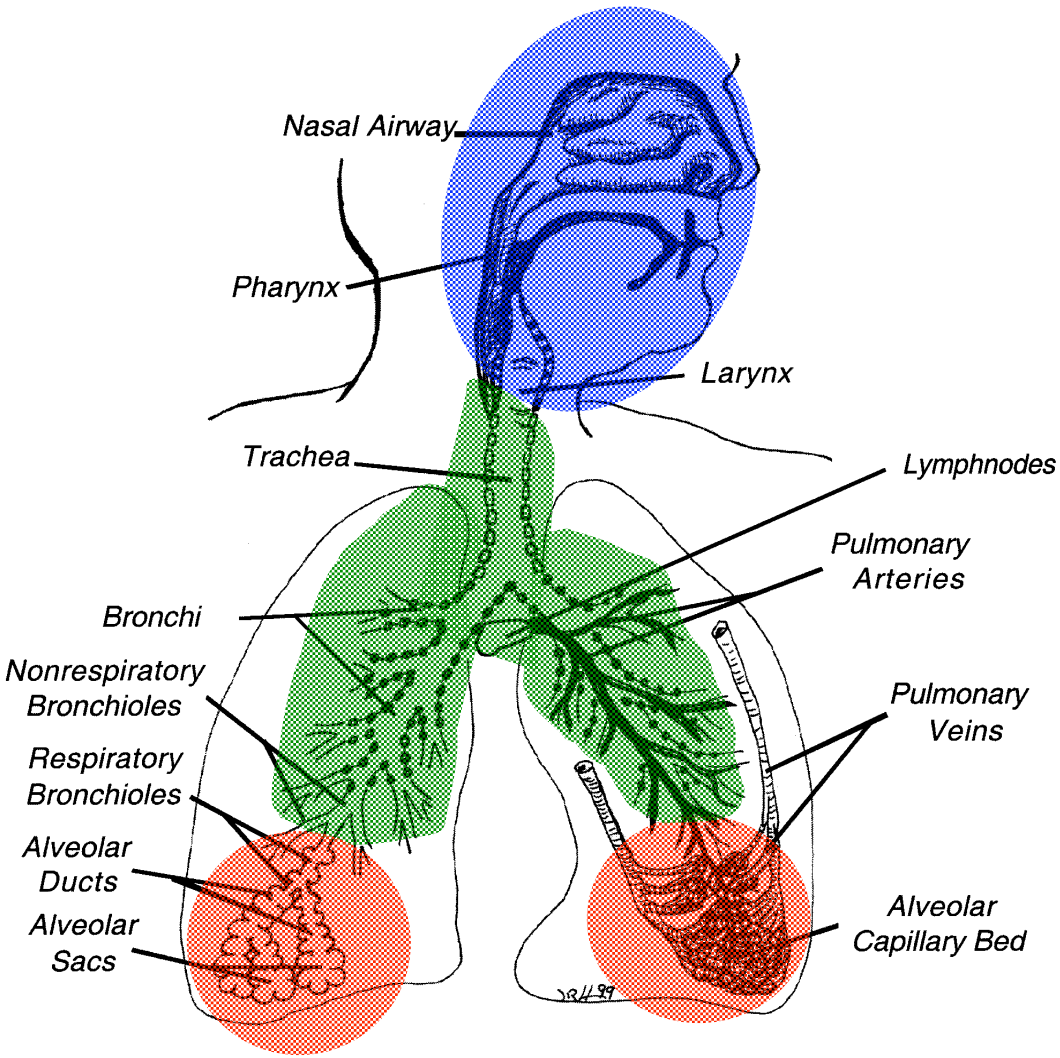
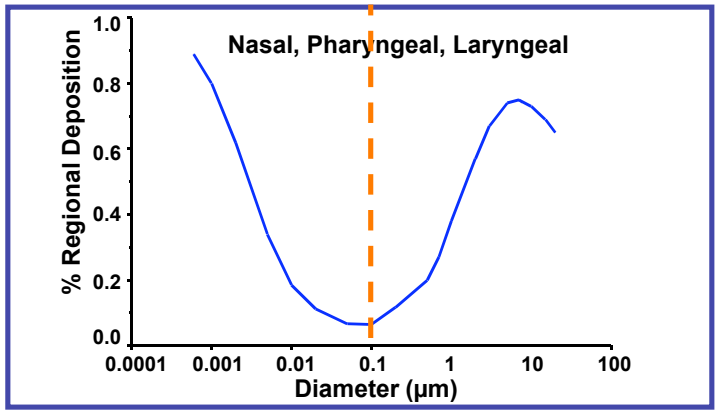
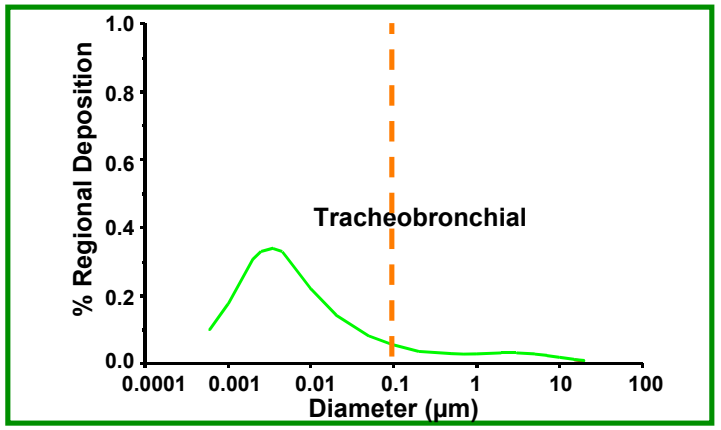

S
L
H
T
Response
toxic
beneficial
0
0
Exposure or Dose
L – linear no threshold
S - supralinear
T- threshold
H – hormetic
(biphasic)

Figure 8


Nasal Airway
Pharynx
Larynx
Trachea
Lymphnodes
Pulmonary Arteries
Bronchi
Nonrespiratory Bronchioles
Respiratory Bronchioles
Alveolar Ducts
Alveolar Sacs
Pulmonary Veins
Alveolar Capillary Bed


Nasal, Pharyngeal, Laryngeal
% Regional Deposition
1.0
0.8
0.6
0.4
0.2
0.0
0.0001
0.001
0.01
0.1
1
10
100
Diameter (µm)


Tracheobronchial
% Regional Deposition
1.0
0.8
0.6
0.4
0.2
0.0
0.0001
0.001
0.01
0.1
1
10
100
Diameter (µm)

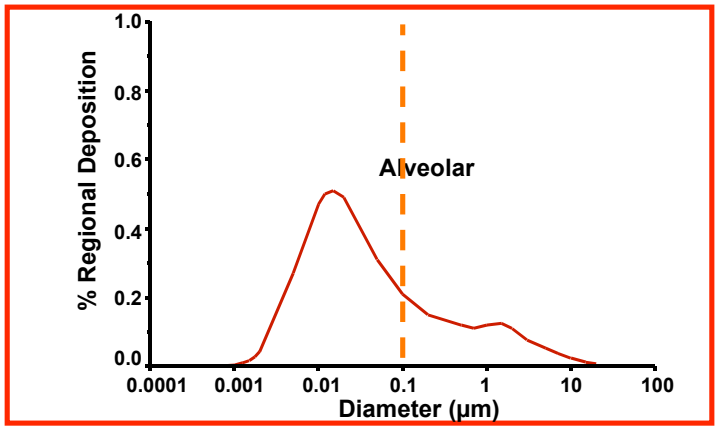

Alveolar
% Regional Deposition
1.0
0.8
0.6
0.4
0.2
0.0
0.0001
0.001
0.01
0.1
1
10
100
Diameter (µm)

Figure 9

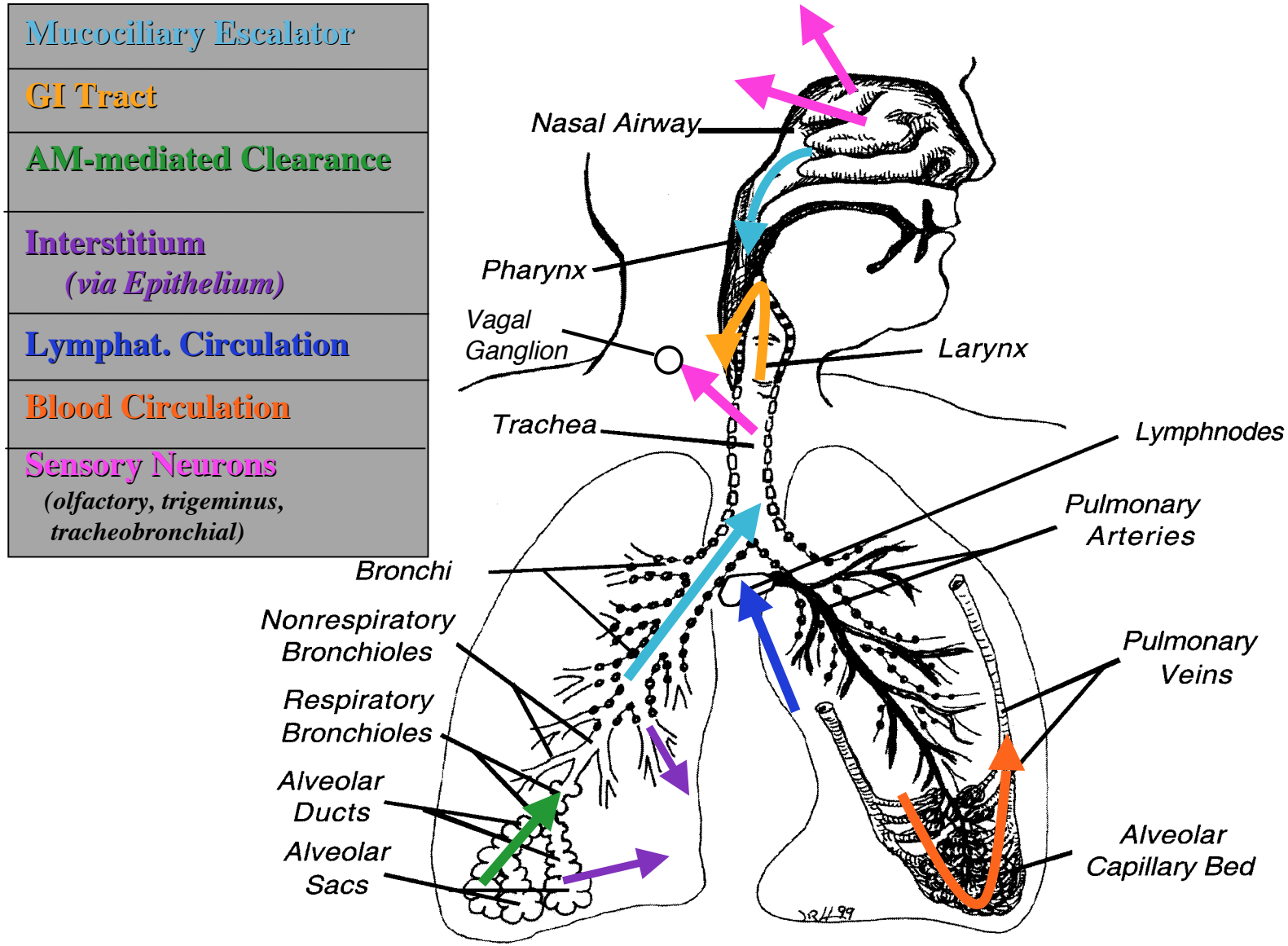

Mucociliary Escalator
GI Tract
AM-mediated Clearance
Interstitium
(via Epithelium)
Lymphat. Circulation
Blood Circulation
Sensory Neurons
(olfactory, trigeminus, tracheobronchial)
Nasal Airway
Pharynx
Vagal Ganglion
Larynx
Trachea
Lymphnodes
Pulmonary Arteries
Bronchi
Nonrespiratory Bronchioles
Pulmonary Veins
Respiratory Bronchioles
Alveolar Ducts
Alveolar Sacs
Alveolar Capillary Bed

Figure 10

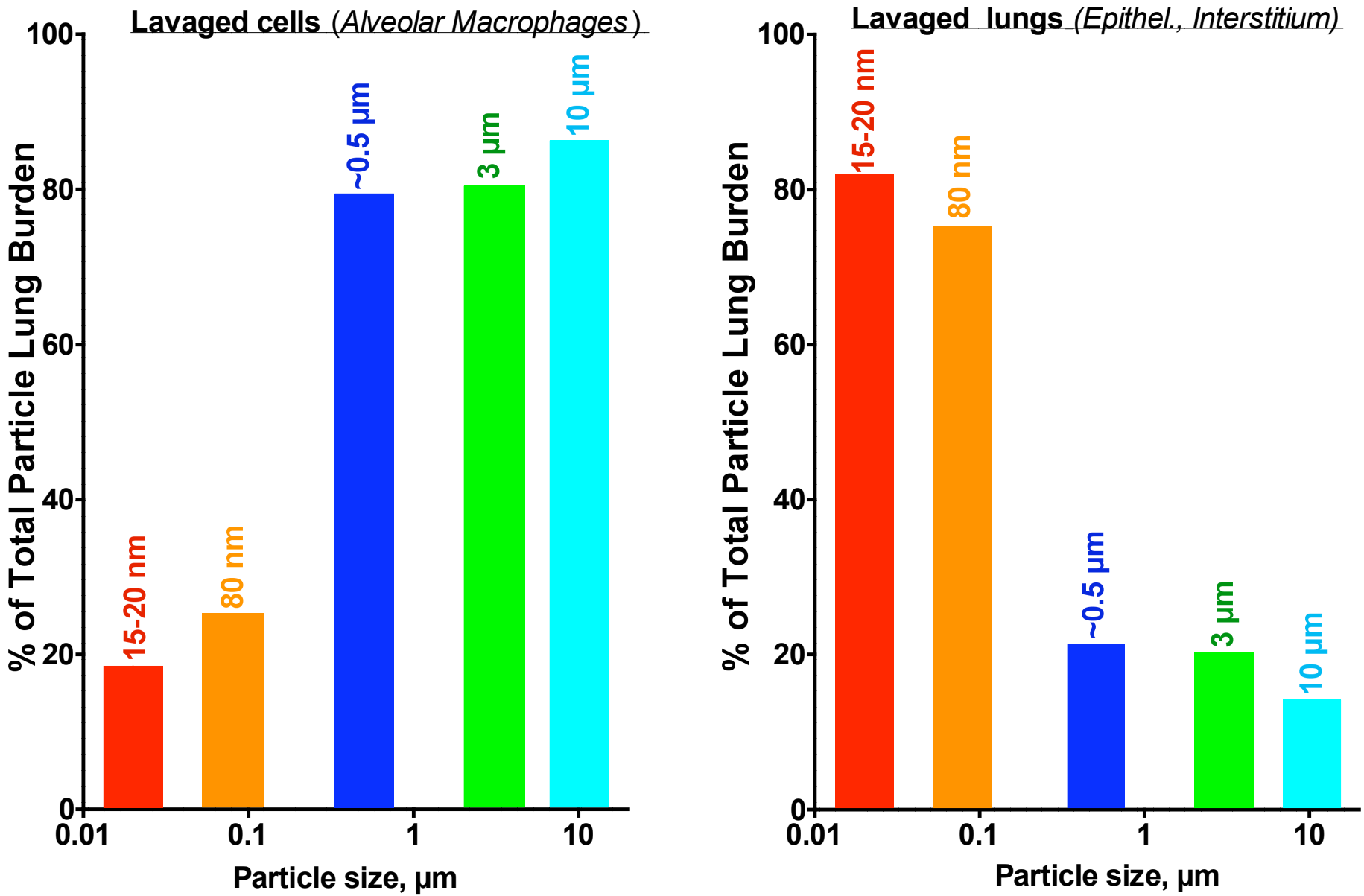
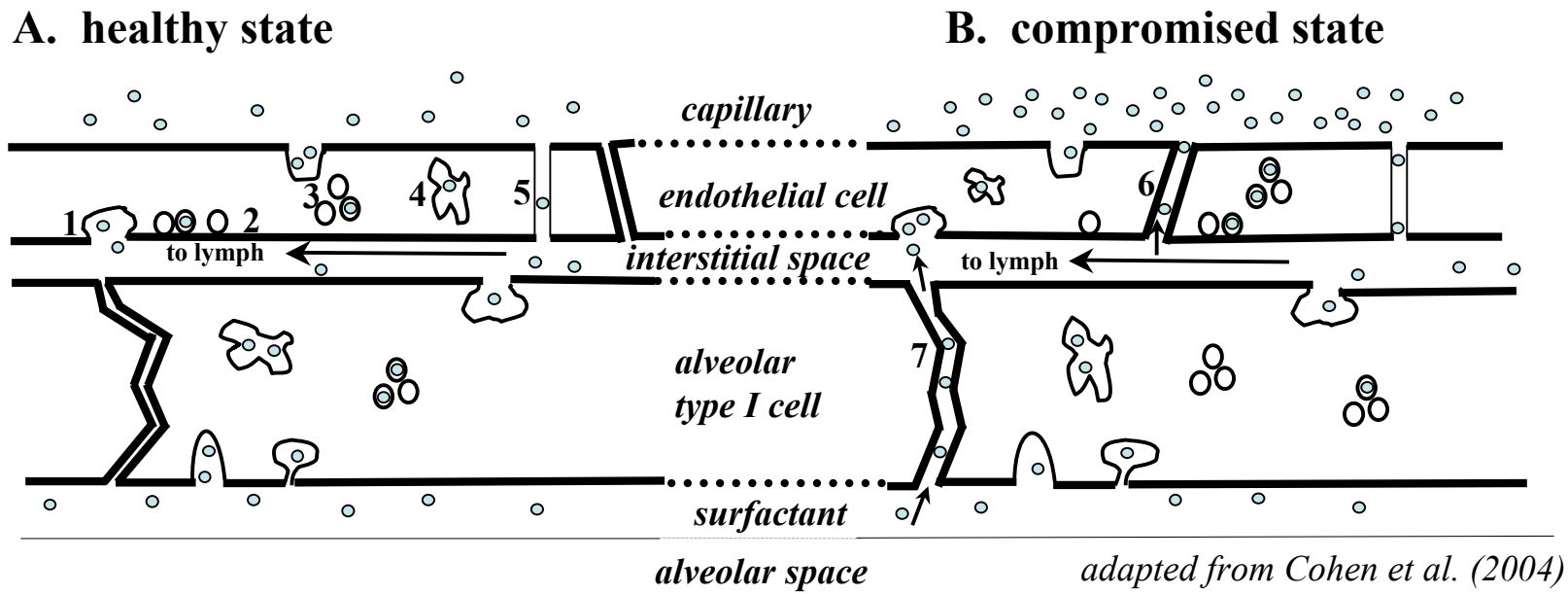

Lavaged cells (Alveolar Macrophages)
% of Total Particle Lung Burden
100
80
60
40
20
0
15-20 nm
80 nm
~0.5 µm
3 µm
10 µm
0.01
0.1
1
10
Particle size, µm
Lavaged lungs (Epithel., Interstitium)
% of Total Particle Lung Burden
100
80
60
40
20
0
15-20 nm
80 nm
~0.5 µm
3 µm
10 µm
0.01
0.1
1
10
Particle size, µm

Figure 11



adapted from Cohen et al. (2004)

1 = traditional caveolae
2 = static caveolae
3 = mobile caveolae
4 = caveosome
5 = transcellular channel

caveolae

6 = capillary endothelial tight junctions
7 = alveolar epithelial cell tight junction
○ = nano-sized particle

Figure 12

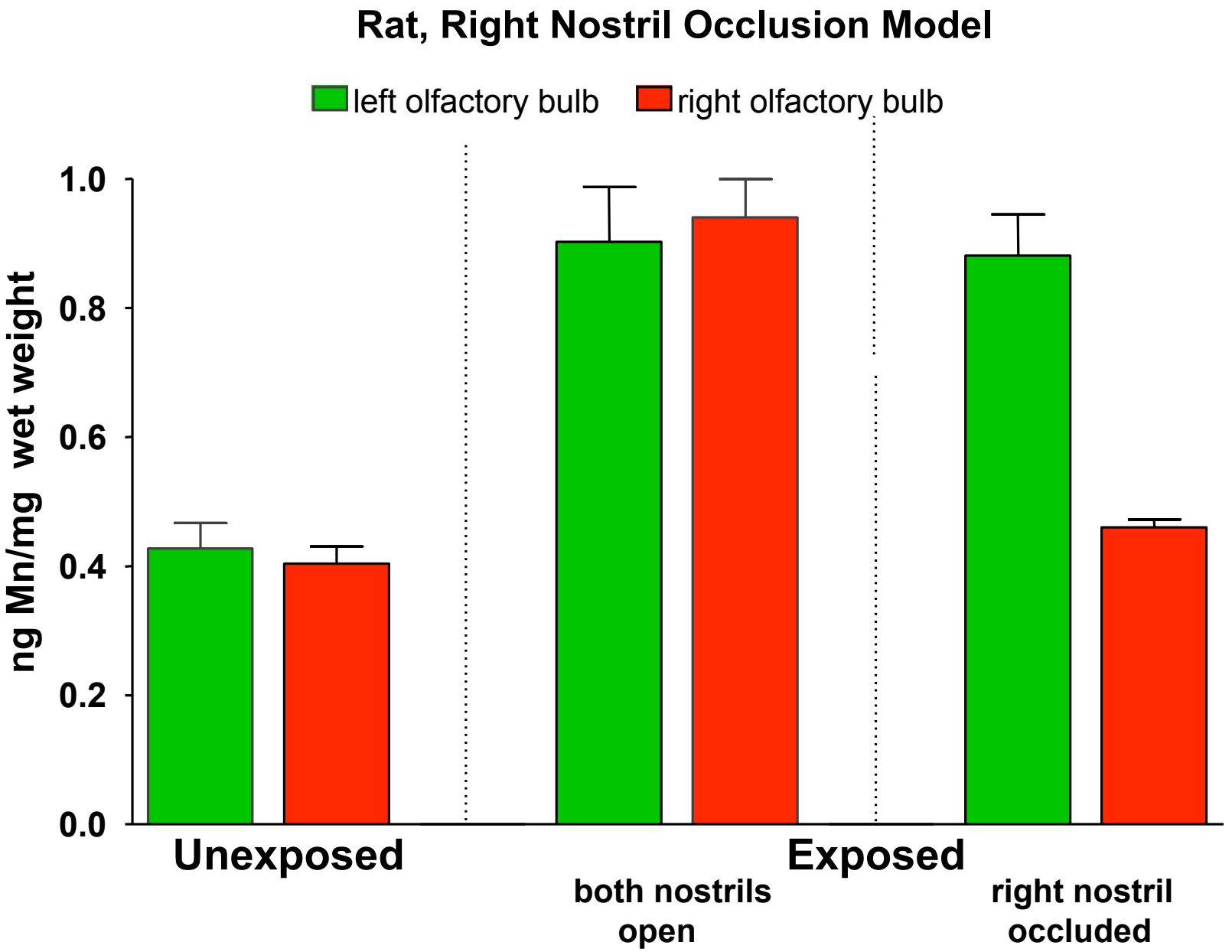
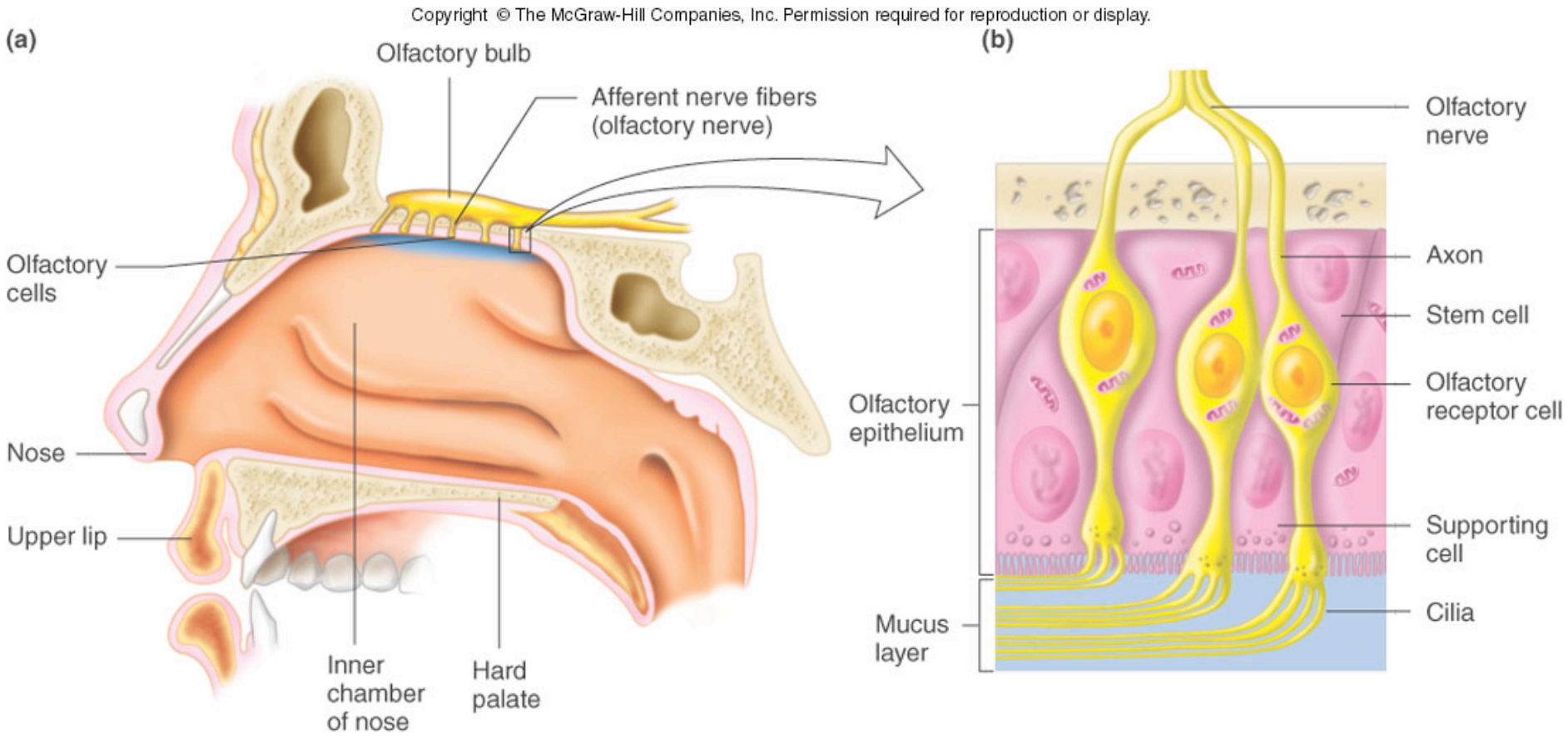

Rat, Right Nostril Occlusion Model
left olfactory bulb
right olfactory bulb
ng Mn/mg wet weight
1.0
0.8
0.6
0.4
0.2
0.0
Unexposed
Exposed
both nostrils open
right nostril occluded

Figure 13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(a)
Olfactory bulb
Afferent nerve fibers (olfactory nerve)
Olfactory cells
Nose
Upper lip
Inner chamber of nose
Hard palate
(b)
Olfactory nerve
Axon
Stem cell
Olfactory receptor cell
Olfactory epithelium
Supporting cell
Cilia
Mucus layer

Figure 14

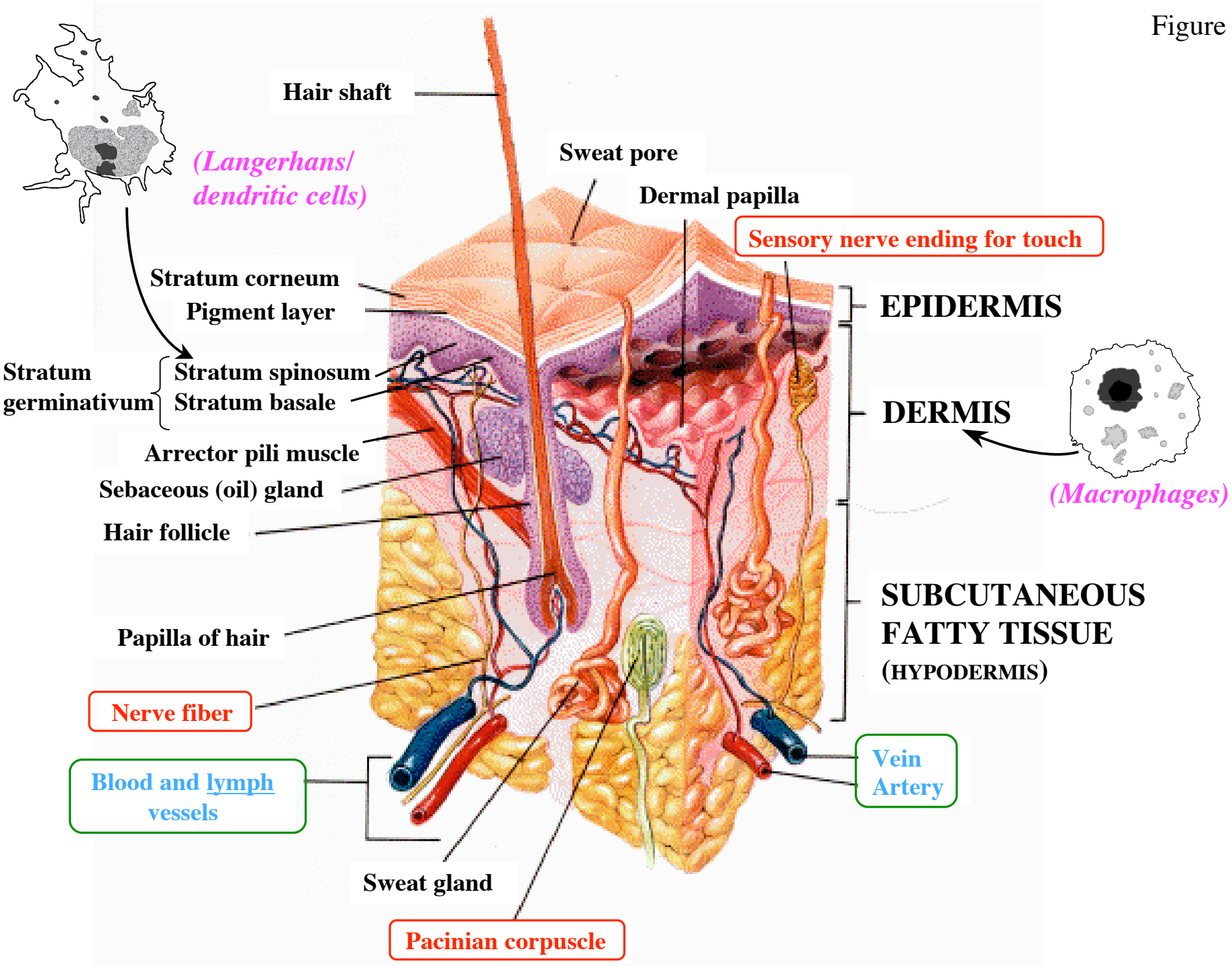
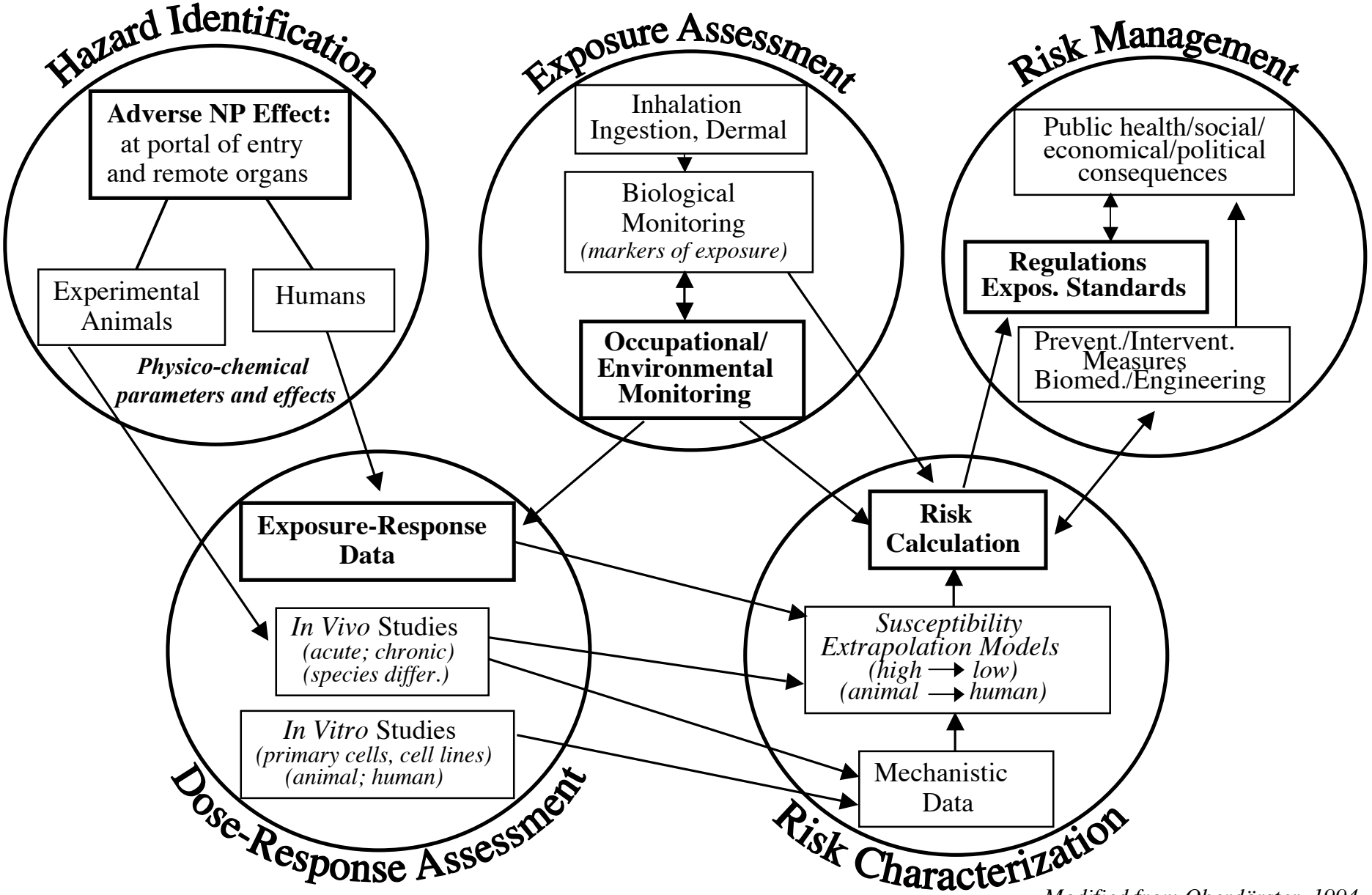

Hair shaft
(Langerhans/ dendritic cells)
Sweat pore
Dermal papilla
Sensory nerve ending for touch
Stratum corneum
Pigment layer
EPIDERMIS
Stratum germinativum
Stratum spinosum
Stratum basale
DERMIS
(Macrophages)
Arrector pili muscle
Sebaceous (oil) gland
Hair follicle
SUBCUTANEOUS FATTY TISSUE (HYPODERMIS)
Papilla of hair
Nerve fiber
Blood and lymph vessels
Vein
Artery
Sweat gland
Pacinian corpuscle

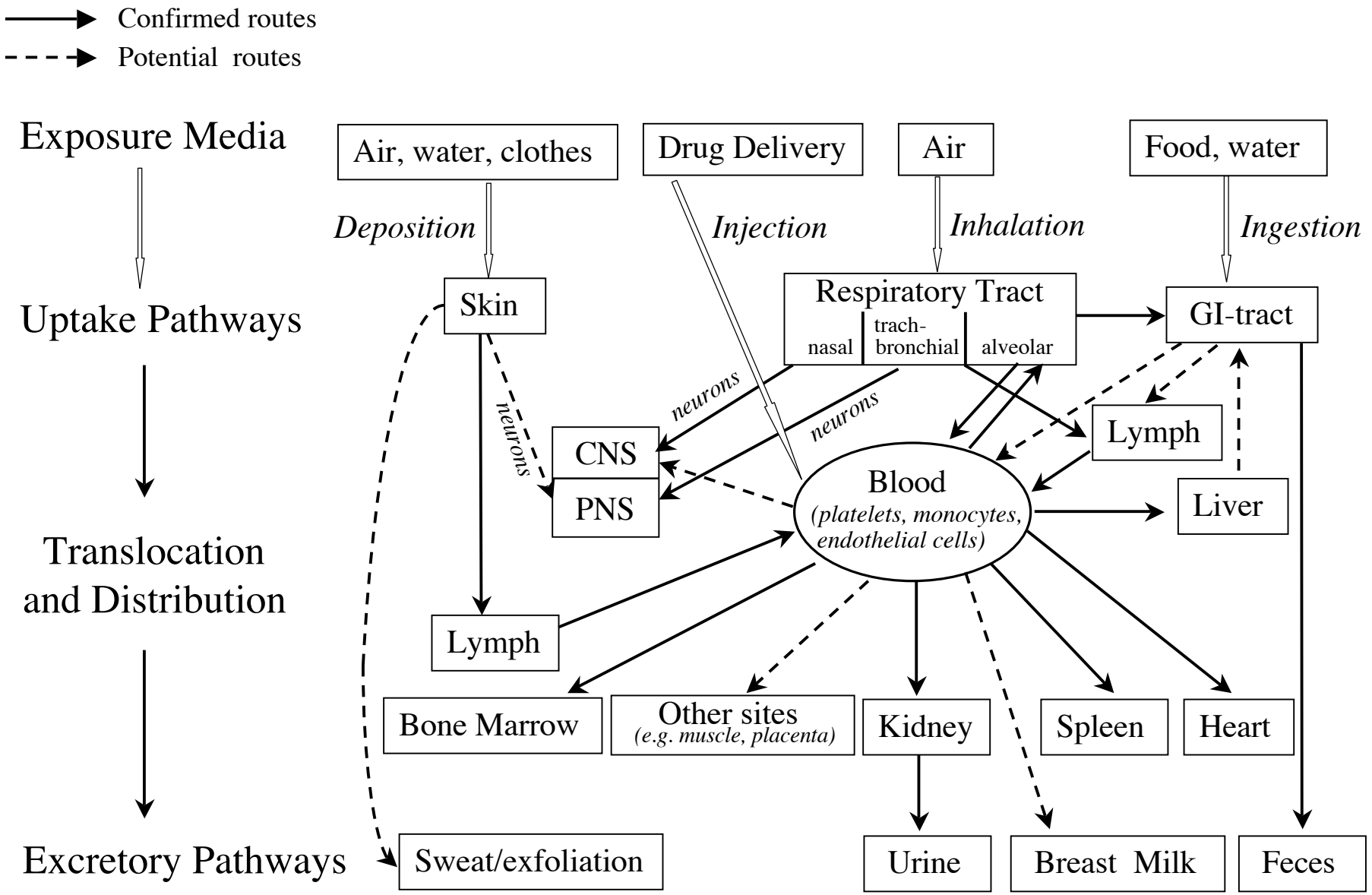
Figure 15

Risk Assessment and Risk Management Paradigm For Engineered Nanoparticles (NP)



Modified from Oberdörster, 1994

Figure 16


Confirmed routes
Potential routes
Exposure Media
Uptake Pathways
Translocation and Distribution
Excretory Pathways
Air, water, clothes
Drug Delivery
Air
Food, water
Deposition
Injection
Inhalation
Ingestion
Skin
Respiratory Tract
nasal
trach-bronchial
alveolar
GI-tract
neurons
neurons
neurons
CNS
PNS
Lymph
Blood
(platelets, monocytes, endothelial cells)
Liver
Lymph
Bone Marrow
Other sites
(e.g. muscle, placenta)
Kidney
Spleen
Heart
Sweat/exfoliation
Urine
Breast Milk
Feces